Basic study on wakame assimilative immunobiotics for immunosynbiotic application

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Basic study on wakame assimilative immunobiotics for immunosynbiotic application

ワカメ資化性イムノバイオティクスの

イムノシンバイオティクスとしての利用性に関する基礎研究

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食品機能健康科学講座

動物資源化学分野

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Chapter 1
Introduction

Fermented milk products are consumed widely in the world due to some beneficial effects. In the early 20th century, Elie Metchnikoff, Russian-born zoologist, and microbiologist who received the 1908 Nobel Prize for Physiology or Medicine with Paul Ehrlich presumed that aging was caused by particular harmful bacterial strains in the gut and, that some food such as sour milk, yogurt and kefir containing lactic acid-producing bacteria could prolong the life[1]. Meanwhile, Louis Pasteur first discovered fermentation by lactic acid bacteria and Henry Tissier, a French paediatrician, isolated a Y-shaped bacterium for intestinal microbiota of breast-fed infants and named it "bifidus"[2]. Depending on their works, people became interested on those functional bacteria till the term "probiotics" was put forward and it came into common use after 1980.

The term "probiotics", which was first introduced by Lilly and Stillwell in 1965, was defined as growth-promoting factors produced by microorganisms that stimulate the growth of other organisms, in contrast to antibiotics[3]. In 1989, probiotics were redefined by Roy Fuller as living bacteria that have beneficial effects on the host by improving the balance of microflora in the intestine[4], which has been broadened to state that "a probiotic is a mono- or mixed culture of live microbes which, when applied to animal or man, affect the host beneficially by improving the properties of the indigenous microflora"[5]. The definition of probiotics has been modified la. In 2001, a joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO)

expert consultation on health and nutritional properties of powder milk with live lactic acid bacteria redefined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host“, emphasizing on the importance of the viability and an adequate dose of probiotic bacteria in order to exert the desirable effects[6]. However, many studies indicated that the dead cells of probiotics microorganisms also generate beneficial biological responses, while the components of dead cells induce an anti-inflammatory response in the host intestine[7]. Since the use of dead probiotics has several attractive advantages (e.g. safety and long shelf-life), redefinition or a new definition would be needed for the coming products of probiotics in the future. After the proposition of this definition, many terms related to probiotics have been put forward reflecting the great advances in the science of probiotics, which is becoming increasingly complex.

Immunobiotics were identified as those probiotic bacteria that promote health through the beneficial modulation of the mucosal immune system[8]. Recently immunobiotics were reported to have the capacity of beneficially modulating the intestinal activation of Toll-like receptor 3 (TLR3) and to reduce the local inflammatory tissue damage indicating their ability to influence antiviral immune responses[9]. In addition, immunobiotics had been shown to possess specific effects in the modulation of the intestinal immunity against rotavirus and it was emphasized that their immunomodulatory functions are species and strain-

specific[10]. One of the most common sources in the search for probiotic strains is the population of microorganisms that colonize mucosal surfaces such as the gastrointestinal tract. In this regard, as described in several animal species, the porcine gastrointestinal tract contains a rich and varied microbial population designated as the microbiota, which has a symbiotic relationship with the host, including the porcine host[11]. In contrast the gastrointestinal tract infections caused by pathogens such as *Escherichia coli*, *Salmonella* ssp., and *Clostridium perfringens* have been considered as major health problems in weaning piglets, and have caused a severe economic loss on farms and food safety problems[12]. Then, the porcine intestinal microbiota is an interesting source of potential probiotic bacteria for their application in the pig industry. In 2012, 70% of antibiotics were consumed by animals and it was estimated that by 2030, global consumption of antibiotics in livestock production would be increased by two-third, and researches over the world are urged to search for effective alternatives[13]. Here, we proposed that “immunosynbiotics”, a combination of immunobiotics and immunoprebiotics with synergistic effects when used together in feed, would be one of the most promising candidates for substitutes of antibiotics to solve the severe problem of antibiotic resistance in the world[14].

Prebiotics, such as seaweed, are defined as indigestible food ingredients that have a beneficial effect on the host by selectively stimulating the growth and/ or activity of one or a limited number of bacterial species, which are already

present in the intestinal tract, and thus improve host health[15]. Seaweed has a variety of biologically active components that could modulate the immune system function, including anti-inflammatory and antiviral activities[16]. Immunoprebiotics are prebiotics that have positive immunomodulatory effects in the host. One example of immunoprebiotics is wakame (*Undaria pinnatifida*)[17]. Wakame contains indigestible polysaccharides such as alginic acid, cellulose, hemicellulose, and fucoidan. Only some bacteria have the ability to utilize degraded polysaccharides[17]. The addition of wakame to feed proved to increase the percentage of peripheral blood NK cells and $CD3^+CD4^-CD8^+$ lymphocytes, and also alter microbiota in the porcine large intestine[18]. Thus, as the abundant resource of probiotics, the intestine of porcine may be the habitat of wakame assimilative probiotics. Here, we used two parts of wakame, leaf and stalk, to evaluate the wakame assimilative ability of potential probiotic strains in enzyme-treated wakame broth, which was previously developed by our group, with the intention of developing immunosynbiotic food and feed supplements.

The *in vitro* selection of potential immunobiotic strains can be a great challenge, particularly when there are no appropriate laboratory tools to evaluate their beneficial effect directed at a specific host species. In this regard, we have previously established a porcine intestinal epithelial (PIE) cell line that demonstrated to be remarkably useful *in vitro* tool for the screening and selection of immunomodulatory lactobacilli. The PIE cell system allows the efficient

selection of potential immunobiotics strains with the ability to differentially regulate innate immune responses in the intestinal mucosa triggered by the activation of Pattern Recognition Receptors (PRRs) such as TLR3 and TLR4[19].

On the other hand, the gastrointestinal (GI) epithelium is frequently exposed to digestive enzymes, fecal material, microorganisms and their products in the lumen. There is a layer of mucus lining on the GI tract, which plays an important role in lubricating and protecting the epithelium from mechanical and chemical stress. The mucus layer is the first line against luminal substances, especially the commensal bacteria and invading pathogens, providing a physical and semi-permeable barrier between the epithelium and the contents of the lumen. The thickness of the layer is around 400 μm in the intestine[20], which contains 2 layers[21]. The thinner inner mucus layer is difficult to dislodge, whereas the outer layer mainly consists of secreted mucins, nonspecific antimicrobials and specific antimicrobial immunoglobulins[22].

Mucins, the major protein components of the mucus, are glycoproteins with high molecular weight from 0.5×10^6 Daltons to 25×10^6 Daltons. The mucin secretion is mainly by goblet cells in the surface epithelium in the GI tract[23]. The formation of mucin starts in endoplasmic reticulum with the production of the protein backbones, which comprises tandem repeats rich in threonine, serine and/or proline residues. After that, the oligosaccharides are highly attached to the variable number tandem repeats (VNTR) of the backbones

by O-linked glycosylation range from 1 to more than 20 residues in the Golgi apparatus (Fig. 1-1). Moreover, N-acetylneuraminic acid (Neu5Ac, the predominant sialic acid found in mammalian cells) and sulfate residues are added to the end of the oligosaccharides. The oligosaccharide structures can be linear or branched, and acidic or neutral in nature, which shows a huge variation in different parts of the GI tract[24, 25].

The mucus layer also provides a habitat for microbiota. This first point mucosal surface contact is a prerequisite for the colonization and persistence for probiotics in the GI tract and provides a competitive advantage in this ecosystem. In fact, interactions between some lactobacillus strains and carbohydrate moieties of glycoconjugates, including glycolipids and mucins have been reported. Some *Lactobacillus* strains express adhesins on their cell surface that mediates attachment to the mucus layer[26]. The adhesion to mucin is considered to be an important property of probiotic lactobacilli since it is the first step for the colonization of the host´s mucosa. The high adhesion ability of probiotic lactobacilli can improve the gut residence time of lactobacilli, and is involved in the exclusion of pathogens and the protection of epithelial cells.

On the basis of the above background, this work aimed to select and characterize potentially beneficial lactobacilli strains isolated from the intestine of wakame-fed pigs by evaluating their interaction with PIE cells in terms of their ability to regulate TLR3- or TLR4-mediated innate immune responses, as well as

their adhesion capabilities. In addition, their capacity of wakame assimilation and tolerance to gastric juice was assessed. These functional studies were complemented with a comparative genomic evaluation using the complete genome sequences of porcine lactobacilli that demonstrated interesting probiotic/ immunobiotic properties. The ultimate objective of this work is the selection of highly efficient immunobiotic strains that combined with wakame as a prebiotic, could be used in the development of promising immunosymbiotics as substitutes for antimicrobials in both feed and food industries.

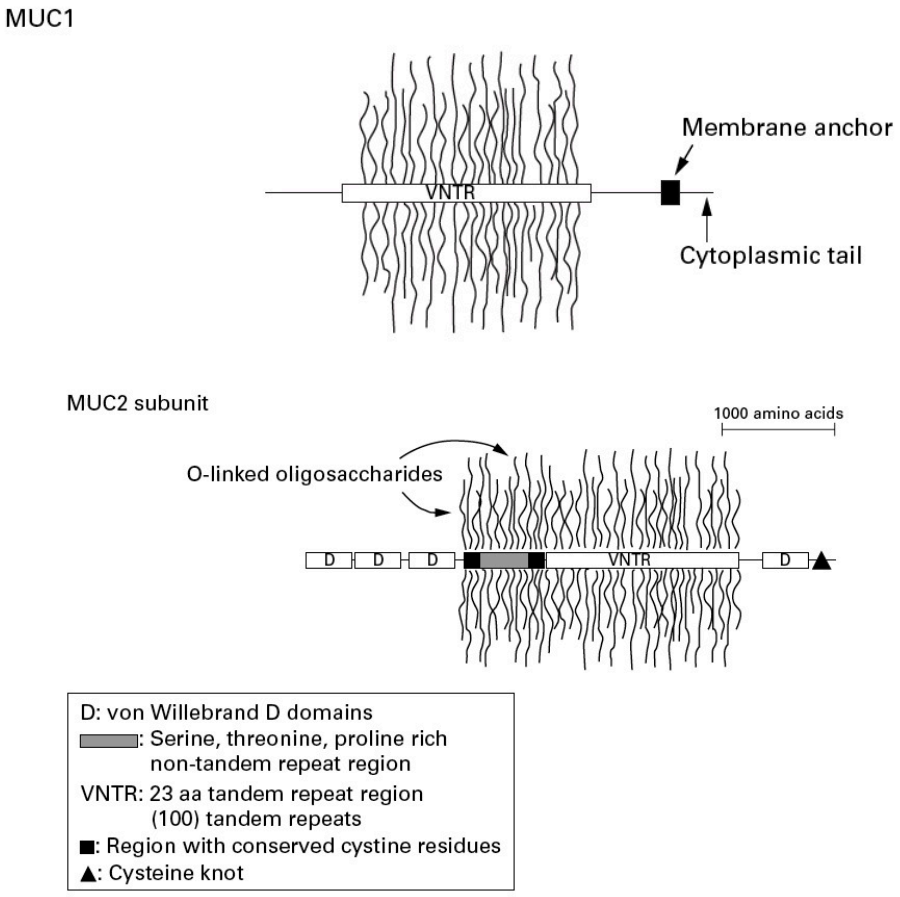


Figure 1-1. The structure of MUC1 and MUC2.

Chapter 2

Immunomodulatory response of isolated lactobacilli and genomic studies

2.1. Introduction

The innate immune system in mammals is composed of functional modules, that evolved to provide different forms of protection against pathogens. Microorganisms including bacteria and virus would trigger the activation of antimicrobial defenses and stimulate the adaptive immune response through pattern recognition receptors (PRRs)[27]. Several classes of PRRs, including Toll-like receptors and cytoplasmic receptors, could recognize distinct microbial components and directly activates immune cells[28]. Toll-like receptors could recognize pathogen-associated molecules, such as lipoteichoic acid (recognized by TLR2) and lipopolysaccharide (TLR4)[29]. TLR3 is activated by double-stranded RNA (dsRNA) related to viral infection[30]. Thus, TLR3 activates genes for secreted antiviral cytokines, such as interferon (IFN-β) and proteins encoding intracellular, viral, stress-inducible proteins[31]. TLR4 has two adapter complexes: TIRAP– MyD88, which drives the induction of inflammatory cytokines, and TRAM–TRIF, which induces type I IFN as well as inflammatory cytokines[32].

In the gastrointestinal tract, the epithelia lying over the mucosal surface, where intestinal epithelial cells (IECs) participate in the regulation of the mucosal immune response to pathogens by interacting with the immune cells in Peyer's patches, lymphoid tissue and intraepithelial lymphocytes[33]. IECs express PRRs that recognize structural components, exogenous pathogen-associated molecular patterns (PAMPs) possessed by microorganism[28]. Cytokines and chemokines are

expressed by immune cells and also IECs. Cytokines, including IL-1α, -1β, -6, -8, TNF-α and MCP-1, expressed also by normal epithelial cells are up-regulated in response to microbial infection[34]. In this study, we used porcine intestinal epitheliocytes (PIE) previously established by our group, which has been confirmed to be efficient laboratory tool for the evaluation of the immunomodulatory effects of immunobiotics in the context of Enterotoxigenic *Escherichia coli* (ETEC)[35] and poly(I:C)[36] stimulations.

In this chapter, we aimed to select promising candidates of immunobiotics from lactobacilli strains isolated from the intestine of wakame-fed pigs. The immunomodulatory potential effects of lactobacilli were evaluated by pre-stimulating PIE cells with the microorganisms and evaluating the expression changes of IFN-β, Mx1, IL-8, and MCP-1 after the challenged with ETEC or poly(I:C). Though lactobacilli belonging to the same species and isolated from the same origin may have a different immunomodulatory response. Then, the functional studies were complemented with genomics analysis in order to figure out the critical genetic factors related to the different immunomodulatory effects.

2.2. Materials and Methods

2.2.1. Bacteria culture condition

L. salivarius strains were isolated from the mucus membrane of the small intestine (jejunum, jejunum Peyer's patches, ileum, and ileum Peyer's patches) of wakame-fed pigs by using originally developed wakame-based mediums as described previously[14]. The lactobacilli strains and their isolation origin evaluated in this chapter are listed in Table 2-1. The *L. salivarius* strains isolated from the intestinal tract of wakame-fed pigs were designated as FFIG.

For the experiments of this work, lactobacilli strains were grown in Man-Rogosa-Sharpe (MRS) broth at 37 °C. For the in vitro immunomodulatory assays, overnight cultures were harvested by centrifugation, washed three times with sterile phosphate-buffered saline (PBS), counted in a Petroff-Hausser counting chamber, and resuspended in DMEM until use.

2.2.2. PIE Cells

The PIE cell line was originally established at Tohoku University from the intestinal epithelia of an unsuckled neonatal pig, as described previously[37 38]. DMEM medium supplemented with 10% fetal calf serum (FCS), penicillin (100 mg/mL), and streptomycin (100 U/mL) was used for the maintenance of PIE cells. The cells (3.0×10^4 per well) were grown in 12 well type I collagen-coated plates at 37 °C in a humidified atmosphere of 5% CO_2. After three days of culturing

period, 1 mL of DMEM containing the different *L. salivarius* strains isolated from the intestine of wakame-fed pigs (5×10^7 cells/mL) were added to PIE cells monolayers. Cells were further incubated for 48 h at 37 °C, 5% CO_2. PIE cells were washed with fresh medium to eliminate lactobacilli and subsequently stimulated with 10 μg/mL of poly(I:C) (Sigma Aldrich, St. Louis, MI, USA) or enterotoxigenic *Escherichia coli* (ETEC) for 12 h, to induce the activation of TLR3 and TLR4, respectively. The expressions of IFN-β and Mx1 were evaluated after TLR3 activation, while the expression of IL-8 and MCP-1 were studied after TLR4 stimulation.

2.2.3. RT-qPCR

The expression of immune factors in PIE cells was studied as described previously[39]. Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and its purity and quantity were analyzed by a NanoDrop spectrophotometer ND-1000 UV-Vis (NanoDrop Technologies, Wilmington, DE). The RNA (500 ng) was used to synthesize cDNA by Thermal cycler (BIO-RAD, Hercules, California, USA) with the Quantitect reverse transcription (RT) kit (Qiagen, Tokyo, Japan) following the manufacturer instructions. The qPCR was performed in a 7300 real-time PCR system (Applied Biosystems, Warrington, UK) with platinum SYBR green (qPCR supermix uracil-DNA glycosylase with 6-carboxyl-X-rhodamine, Invitrogen). For the PCR reaction, 2.5 μL of cDNA was

mixed with 7.5 μL of the master mix that included RT enzyme, SYBR green, forward, and reverse primers (1 pmol/μL). The reaction cycles were performed as follow: 50 °C for 5 min; 95 °C for 5 min; 40 cycles at 95 °C for 15 s, 60 °C for 30 s and finally 72 °C for 30 s. β-actin was used as a housekeeping gene because of its high stability across various porcine tissues. The expression of the housekeeping gene was used to normalize cDNA levels for differences in total cDNA levels in the samples.

Table 2-1. *Ligilactobacillus salivarius* strains evaluated in this study[14].

No.	Strains	Species	Origin (porcine intestine)
1	FFIG17	*Ligilactobacillus salivarius*	ileum peyer's patch
2	FFIG18	*Ligilactobacillus salivarius*	ileum peyer's patch
3	FFIG19	*Ligilactobacillus salivarius*	ileum peyer's patch
4	FFIG20	*Ligilactobacillus salivarius*	ileum peyer's patch
5	FFIG21	*Ligilactobacillus salivarius*	ileum peyer's patch
6	FFIG22	*Ligilactobacillus salivarius*	ileum peyer's patch
7	FFIG23	*Ligilactobacillus salivarius*	ileum peyer's patch
8	FFIG24	*Ligilactobacillus salivarius*	ileum peyer's patch
9	FFIG26	*Ligilactobacillus salivarius*	ileum peyer's patch
10	FFIG27	*Ligilactobacillus salivarius*	ileum peyer's patch
11	FFIG28	*Ligilactobacillus salivarius*	ileum peyer's patch
12	FFIG29	*Ligilactobacillus salivarius*	ileum peyer's patch
13	FFIG30	*Ligilactobacillus salivarius*	ileum peyer's patch
14	FFIG31	*Ligilactobacillus salivarius*	ileum peyer's patch
15	FFIG32	*Ligilactobacillus salivarius*	ileum peyer's patch
16	FFIG33	*Ligilactobacillus salivarius*	ileum peyer's patch
17	FFIG34	*Ligilactobacillus salivarius*	ileum peyer's patch
18	FFIG35	*Ligilactobacillus salivarius*	ileum peyer's patch
19	FFIG36	*Ligilactobacillus salivarius*	ileum peyer's patch
20	FFIG37	*Ligilactobacillus salivarius*	ileum peyer's patch
21	FFIG38	*Ligilactobacillus salivarius*	ileum peyer's patch
22	FFIG39	*Ligilactobacillus salivarius*	ileum peyer's patch
23	FFIG40	*Ligilactobacillus salivarius*	ileum peyer's patch
24	FFIG41	*Ligilactobacillus salivarius*	ileum peyer's patch
25	FFIG42	*Ligilactobacillus salivarius*	ileum peyer's patch
26	FFIG43	*Ligilactobacillus salivarius*	ileum peyer's patch
27	FFIG44	*Ligilactobacillus salivarius*	ileum peyer's patch
28	FFIG45	*Ligilactobacillus salivarius*	ileum peyer's patch

29	FFIG46	*Ligilactobacillus salivarius*	ileum peyer's patch
30	FFIG47	*Ligilactobacillus salivarius*	jejunum
31	FFIG48	*Ligilactobacillus salivarius*	jejunum
32	FFIG49	*Ligilactobacillus salivarius*	jejunum
33	FFIG50	*Ligilactobacillus salivarius*	jejunum
34	FFIG51	*Ligilactobacillus salivarius*	jejunum
35	FFIG52	*Ligilactobacillus salivarius*	jejunum
36	FFIG53	*Ligilactobacillus salivarius*	jejunum
37	FFIG54	*Ligilactobacillus salivarius*	jejunum
38	FFIG55	*Ligilactobacillus salivarius*	jejunum
39	FFIG56	*Ligilactobacillus salivarius*	jejunum
40	FFIG57	*Ligilactobacillus salivarius*	jejunum
41	FFIG58	*Ligilactobacillus salivarius*	jejunum
42	FFIG59	*Ligilactobacillus salivarius*	jejunum
43	FFIG60	*Ligilactobacillus salivarius*	jejunum
44	FFIG61	*Ligilactobacillus salivarius*	jejunum
45	FFIG62	*Ligilactobacillus salivarius*	jejunum
46	FFIG63	*Ligilactobacillus salivarius*	jejunum
47	FFIG64	*Ligilactobacillus salivarius*	jejunum
48	FFIG65	*Ligilactobacillus salivarius*	jejunum
49	FFIG66	*Ligilactobacillus salivarius*	jejunum
50	FFIG67	*Ligilactobacillus salivarius*	jejunum
51	FFIG68	*Ligilactobacillus salivarius*	jejunum
52	FFIG69	*Ligilactobacillus salivarius*	jejunum
53	FFIG70	*Ligilactobacillus salivarius*	jejunum
54	FFIG71	*Ligilactobacillus salivarius*	jejunum
55	FFIG72	*Ligilactobacillus salivarius*	jejunum
56	FFIG73	*Ligilactobacillus salivarius*	jejunum
57	FFIG74	*Ligilactobacillus salivarius*	jejunum
58	FFIG75	*Ligilactobacillus salivarius*	ileum peyer's patch

59	FFIG76	*Ligilactobacillus salivarius*	ileum peyer's patch
60	FFIG77	*Ligilactobacillus salivarius*	ileum peyer's patch
61	FFIG78	*Ligilactobacillus salivarius*	ileum peyer's patch
62	FFIG79	*Ligilactobacillus salivarius*	ileum peyer's patch
63	FFIG80	*Ligilactobacillus salivarius*	ileum peyer's patch
64	FFIG81	*Ligilactobacillus salivarius*	ileum peyer's patch
65	FFIG82	*Ligilactobacillus salivarius*	ileum
66	FFIG83	*Ligilactobacillus salivarius*	ileum
67	FFIG84	*Ligilactobacillus salivarius*	ileum
68	FFIG85	*Ligilactobacillus salivarius*	ileum
69	FFIG86	*Ligilactobacillus salivarius*	ileum
70	FFIG87	*Ligilactobacillus salivarius*	ileum
71	FFIG88	*Ligilactobacillus salivarius*	ileum
72	FFIG89	*Ligilactobacillus salivarius*	ileum
73	FFIG90	*Ligilactobacillus salivarius*	ileum
74	FFIG91	*Ligilactobacillus salivarius*	ileum
75	FFIG92	*Ligilactobacillus salivarius*	ileum
76	FFIG93	*Ligilactobacillus salivarius*	ileum
77	FFIG94	*Ligilactobacillus salivarius*	ileum
78	FFIG95	*Ligilactobacillus salivarius*	ileum
79	FFIG96	*Ligilactobacillus salivarius*	ileum
80	FFIG97	*Ligilactobacillus salivarius*	ileum
81	FFIG98	*Ligilactobacillus salivarius*	ileum
82	FFIG99	*Ligilactobacillus salivarius*	ileum
83	FFIG100	*Ligilactobacillus salivarius*	ileum
84	FFIG101	*Ligilactobacillus salivarius*	ileum
85	FFIG102	*Ligilactobacillus salivarius*	ileum
86	FFIG103	*Ligilactobacillus salivarius*	ileum
87	FFIG104	*Ligilactobacillus salivarius*	ileum
88	FFIG105	*Ligilactobacillus salivarius*	ileum

89	FFIG106	*Ligilactobacillus salivarius*	ileum
90	FFIG107	*Ligilactobacillus salivarius*	ileum
91	FFIG108	*Ligilactobacillus salivarius*	ileum
92	FFIG109	*Ligilactobacillus salivarius*	ileum
94	FFIG111	*Ligilactobacillus salivarius*	ileum
95	FFIG112	*Ligilactobacillus salivarius*	ileum
96	FFIG114	*Ligilactobacillus salivarius*	ileum
97	FFIG116	*Ligilactobacillus salivarius*	ileum
98	FFIG117	*Ligilactobacillus salivarius*	ileum
99	FFIG118	*Ligilactobacillus salivarius*	ileum
100	FFIG119	*Ligilactobacillus salivarius*	ileum
101	FFIG120	*Ligilactobacillus salivarius*	ileum
102	FFIG121	*Ligilactobacillus salivarius*	ileum
103	FFIG122	*Ligilactobacillus salivarius*	ileum
104	FFIG123	*Ligilactobacillus salivarius*	ileum
105	FFIG125	*Ligilactobacillus salivarius*	ileum
106	FFIG126	*Ligilactobacillus salivarius*	ileum
107	FFIG127	*Ligilactobacillus salivarius*	ileum
108	FFIG128	*Ligilactobacillus salivarius*	ileum
109	FFIG129	*Ligilactobacillus salivarius*	ileum
110	FFIG130	*Ligilactobacillus salivarius*	ileum
111	FFIG131	*Ligilactobacillus salivarius*	ileum
112	FFIG132	*Ligilactobacillus salivarius*	ileum
113	FFIG133	*Ligilactobacillus salivarius*	ileum
114	FFIG134	*Ligilactobacillus salivarius*	ileum
115	FFIG135	*Ligilactobacillus salivarius*	ileum
116	FFIG136	*Ligilactobacillus salivarius*	ileum

2.3. Results

2.3.1. Immunomodulatory response to TLR3 and TLR4 ligands in PIE cells

One-hundred and sixteen *L. salivarius* strains isolated from the jejunum, ileum, or ileum Peyer's patches of wakame-fed pigs (Table 2-1) were evaluated according to their ability to modulate the response of PIE cells to the challenges with TLR3 or TLR4 ligands. The expression changes of IFN-β, Mx1, IL-8, and MCP-1 clearly showed the strain-dependent capacities of *L. salivarius* strains in modulating the innate immune responses in the epithelial cells of porcine origin (Fig. 2-1). Strains like *L. salivarius* FFIG58 and FFIG23 showed a remarkable ability to improve the expression of IFN-β and Mx1 in PIE cells after TLR3 activation. These two strains clustered in different groups, since FFIG58 was also capable of improving MCP-1 expression in ETEC-challenged PIE cells, while the FFIG23 strain did not induce effects in IL-8 or MCP-1 expression after TLR4 activation. *L. salivarius* FFIG53 was also capable of increasing the expression of IFN-β, but no effect was observed for Mx1 (Fig. 2-1). In contrast to FFIG58, FFIG23, and FFIG53, strains such as FFIG60, FFIG63, and FFIG79, significantly reduced the expression of the type I IFN in poly(I:C)-challenged PIE cells. *L. salivarius* FFIG79 not only decreased IFN-β, but also reduced the expression of Mx1, an effect that was not observed for the FFIG60 or FFIG63 strains. The strains FFIG60, FFIG63, and FFIG79 also differed in their abilities to modulate

IL-8 and MCP-1 in ETEC-challenged PIE cells. *L. salivarius* FFIG63 reduced the expression of both inflammatory factors; the FFIG79 diminished only IL-8 expression, while the FFIG60 did not modify the levels of IL-8 or MCP-1 (Fig. 2-1). We also found strains with no ability to influence the response of PIE cells to TRL3 activation, but with the capacity to increase the expression of IL-8 or MCP-1 after ETEC challenge, such as *L. salivarius* FFIG130. In addition, strains such as *L. salivarius* FFIG124 were unable to modulate neither the TLR3- nor the TLR4-mediated responses in PIE cells (Fig. 2-1).

Eight strains were selected due to their differential immunomodulatory abilities for further studies (Fig. 2-1). Even the FFIG strains were isolated from the same origin and under the same environmental pressure (wakame as the main nutritive substrate); they showed different capacities to modulate innate immune responses in PIE cells.

2.3.2. General genomic features of *L. salivarius* isolated from the intestinal tract of wakame-fed pigs

To deepen the characterization of the selected *L. salivarius* strains, their complete genomes were sequenced by Illumina HiSeq. The genomes of *L. salivarius* FFIG58[40], FFIG23, FFIG53, FFIG60, FFIG63, FFIG79, FFIG124, and FFIG130 were sequenced for this work (Table 2-2). The FFIG58 draft genome sequence has an average GC content of 32.9% and a total estimated size of

1,984,180 bp. The other *L. salivarius* strains isolated from the intestinal tract of wakame-fed pigs showed general genomic features that were similar to those found for the FFIG58. The largest genome size was found in *L. salivarius* FFIG23 with 2,041,027 bp, while the smallest genome size was found in the FFIG79 strain with 1,718,597 bp. The average GC content in all the strains ranged between 32.8 and 33.4% (Table 2-2). The clustering of pair-wise average nucleotide identity (ANI) was used as a method to confirm that the FFIG strains belong within the *L. salivarius* species, using a cut-off value of 95% as the species boundary[41]. The heat-map analysis of ANI values of FFIG strains compared with several *L. salivarius* strains clearly indicated that they belong to this species (Fig. 2-2).

To verify that the FFIG isolates were different strains, we constructed phylogenetic trees using the sequences of the 16s rRNA genes (Fig. 2-3) and the Maximum Likelihood method (MLST) analysis with the sequences of the genes *parB*, *rpsB*, *pheS*, *nrdB*, *groEL*, and *ftsQ*[42 43] (Fig. 2-4). In both analyses, the FFIG isolates were compared with *L. salivarius* strains isolated from the intestinal tract of humans (UCC118, REN, HN26-4, NT4-8, and FXJCJ72) or pigs (JCM1046, ZSL006, cp400, KLA006, KLF003, KLW010, and WCA-389-WT-5E), and with available public genomes.

The general genomic characteristics of the FFIGs strains (Table 2-2) were not different from the reported genomes of *L. salivarius* strains available in public databases. However, it was noted that the average genome sizes of the FFIG

strains were smaller than those found for other strains of porcine origin. The genome sizes of *L. salivarius* JCM1046, ZSL006, cp400, KLA006, KLF003, and KLW010 ranged from 1,836,297 to 2,389,395 bp. The number of protein-coding genes ranged from 1,803 in the JCM1046 strain to 2,276 in the KLA006 strain (Table 2-3), while for the FFIG strains, the number of protein-coding genes ranged from 1,726 (FFIG79) to 1,932 (FFIG23) (Table 2-2).

2.3.3. Comparative genomic analysis of "immune phenotype"

According to phylogenetic trees constructed by the Maximum Likelihood method (MLST) analysis with the sequences of the genes *parB*, *rpsB*, *pheS*, *nrdB*, *groEL*, and *ftsQ,* comparative genomic studies were carried out to characterize the *L. salivarius* FFIG strains with different immunomodulatory activities. Thus, we defined four principal "immune phenotypes" according to the ability of the FFIGs strains to modulate the innate immune response in PIE cells after TLR3 or TLR4 activation (Fig. 2-5). Strains with the ability to increase IFN-β and Mx1 and with no remarkable effect on IL-8 and MCP-1 expressions, such as FFIG23 and FFIG53, and strains with the capacity to reduce IFN-β and IL-8 and/or MCP-1, such as FFIG60, FFIG63, and FFIG79 were separated in two "immune phenotype" groups. In addition, the FFIG58 strain capable of increasing IFN-β, Mx1, and MCP-1, and the FFIG130 able to increase IL-8 and MCP-1, but with no effect on IFN-β and Mx1 were considered two "immune phenotype" groups. The

FFIG124 with no effects on the expression of IFN-β, Mx1, IL-8, or MCP-1 in PIE cells was also included as a non-immunomodulatory strain.

The comparative study of four strains (FFIG58, FFIG23, FFIG130, and FFIG79) belonging to each of the "immune phenotype" groups revealed a core-genome of 1276 genes (Fig. 2-5). The strains with the ability to increase IFN-β, *L. salivarius* FFIG58, and FFIG23 had 38 and 200 unique genes, respectively. In addition, the FFIG58 and FFIG23 strains sheared 136 genes that were not found in the FFIG130 or FFIG79 genomes. Among the unique genes of *L. salivarius* FFIG58, we found a peptidoglycan O-acetyltransferase (*patA3*), a septation ring formation regulator (*ezrA*), a membrane protein YdfK (*ydfK*), and an N- acetyl-LL-diaminopimelate aminotransferase (*dapX*). The unique genes for FFIG23 strain included a peptidoglycan O-acetyltransferase (*patA1*), a glycosyltransferase EpsH (*epsH*), and an α-galactosylglucosyldiacylglycerol synthase (*cpoA*). Among the genes sheared by FFIG23 and FFIG58 we found the inner membrane protein YhaI (*yhaI*), the inner membrane transporter YicL (*yicL*), the glycosyltransferase EpsD (*epsD*), the penicillin-binding protein PbpX (*pbpX*), the membrane protein insertase MisCB (*misCB*) and the UDP-N-acetylenolpyruvoylglucosamine reductase (*murB*). *L. salivarius* FFIG130, the strain with the ability to increase IL-8 and MCP-1, had 25 unique genes. In addition, FFIG130 sheared with the FFIG58 strain 20 genes that were not found in FFIG23 or FFIG79 strains (Fig. 2-5). Among the unique genes of FFIG130 there was the peptidoglycan O-

acetyltransferase (*patA5*), the cell division topological determinant MinJ (*minJ*), and an N-acetylmuramoyl-L-alanine amidase domain-containing protein different from the one found in the FFIG58 strain. On the other hand, *L. salivarius* FFIG79 had 80 unique genes that included an inner membrane transport permease YbhR (*ybhR*). In addition, it was observed that FFIG79 sheared with FFIG23 a glycosyltransferase EpsJ (*epsJ*) that was not found in the other *L. salivarius* strains.

The unique genes or the genes sheared by specific strains detected in our comparative genomic analysis are involved in bacterial cell division, in the biosynthesis of the bilayer-forming membrane, the biosynthesis and catabolism of cell-wall peptidoglycan, the biosynthesis of exopolysaccharides (EPS), or are integral inner membrane proteins or involved in the integration of membrane proteins, such as lipoproteins.

To confirm these findings, we further performed a genomic comparison of the strains with the ability to increase IFN-β, *L. salivarius* FFIG58, and FFIG23, with the non-immunomodulatory strain FFIG124 (Fig. 2-6). The comparative study of these three strains revealed a coregenome of 1,521 genes. The strains FFIG58 and FFIG23 had 39 and 59 unique genes, respectively, while they shared 356 genes. *L. salivarius* FFIG124 had 58 unique genes and only sheared 21 genes with the FFIG58. Among the unique genes for the FFIG124 strain, we only found a UDP-N-acetylglucosamine 2-epimerase (*mnaA*). These results further highlight

that the differences in the bacterial surface molecules would determine their ability to modulate immune responses in PIE cells.

These results strongly suggest that the cell wall and the surface molecules expressed in the different FFIG strains are involved in their differential capacity to modulate the immune response of PIE cells.

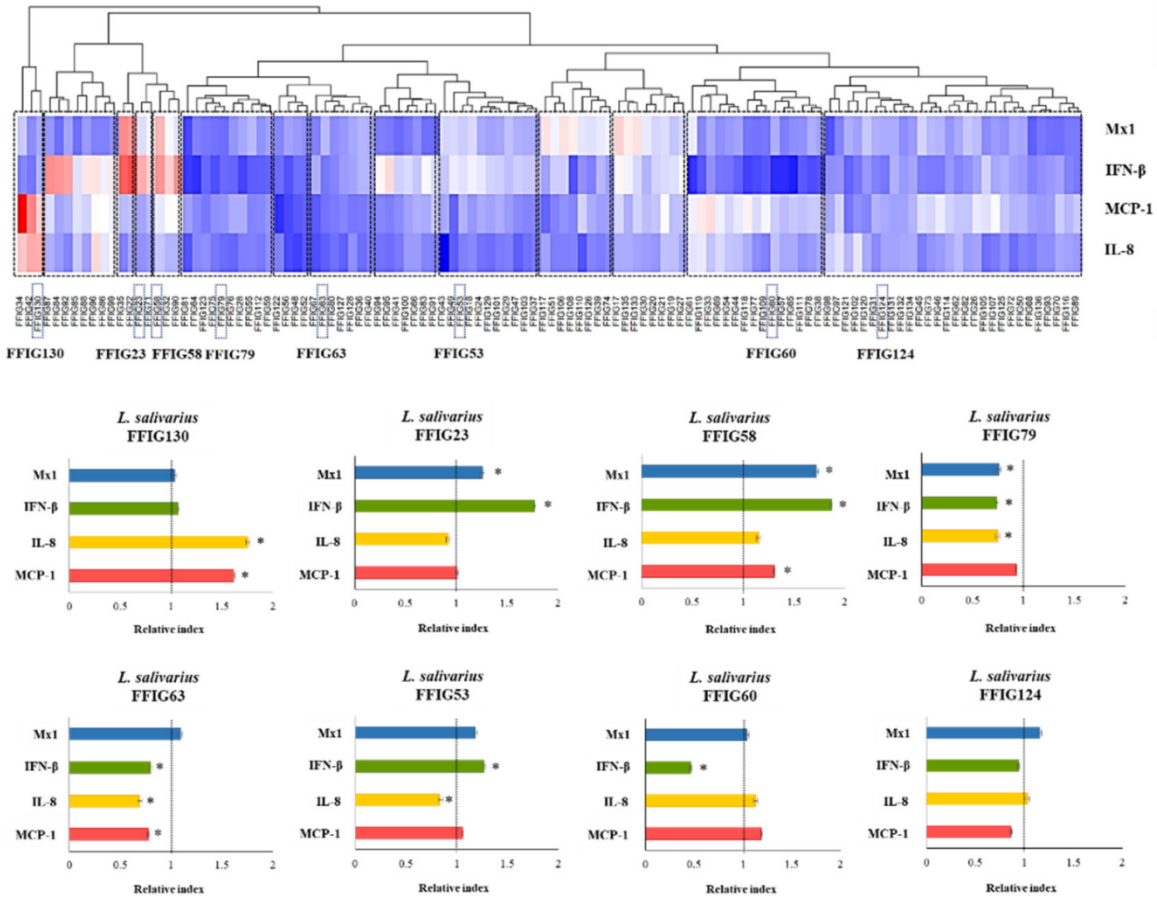


Figure 2-1. Modulation of toll-like receptors (TLRs) mediated innate immune responses in porcine intestinal epithelial (PIE) cells by *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs. PIE cells were stimulated with the different *L. salivarius* strains and challenged with poly(I:C) or enterotoxigenic Escherichia coli (ETEC) to induce the activation of TLR3 and TLR4, respectively. The expressions of interferon (IFN)-β, and the antiviral factor Mx1 were analyzed by qPCR after 12 h of TLR3 activation. The expression of interleukin (IL)-8 and monocyte chemoattractant protein 1 (MCP-1) were analyzed by qPCR after 12 h of TLR4 activation. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to the expression of immune factors in control PIE cells, which were set as one (*$P < 0.05$). Heat-map was constructed considering the fold changes relative to the control PIE cells not treated with lactobacilli and stimulated with poly(I:C) or ETEC.

Table 2-2. Comparison of the general genome features of sequenced *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs. *L. salivarius* genomes are available in the NCBI database, and the accession numbers are provided.

***Ligilactobacillus salivarius* strain**	**Host**	**Sample**	**Genome size (bp)**	**G+C content (%)**	**Protein-coding genes**	**GenBank ID**	**Reference**
FFIG58	*Sus scrofa*	Intestine	1,984,018	32.8	1891	JACBJR000000000.1	[20]
FFIG23	*Sus scrofa*	Intestine	2,041,027	32.8	1932	JACBJS000000000.1	This work
FFIG53	*Sus scrofa*	Intestine	1,948,231	32.9	1863	JACBJT000000000.1	This work
FFIG60	*Sus scrofa*	Intestine	1,948,639	33.0	1855	JACBJU000000000.1	This work
FFIG63	*Sus scrofa*	Intestine	1,777,024	33.3	1786	JACBJV000000000.1	This work
FFIG79	*Sus scrofa*	Intestine	1,718,597	33.4	1728	JACBJW000000000.1	This work
FFIG124	*Sus scrofa*	Intestine	1,806,583	33.2	1812	JACBJX000000000.1	This work
FFIG130	*Sus scrofa*	Intestine	1,862,635	33.1	1778	JACBJY000000000.1	This work

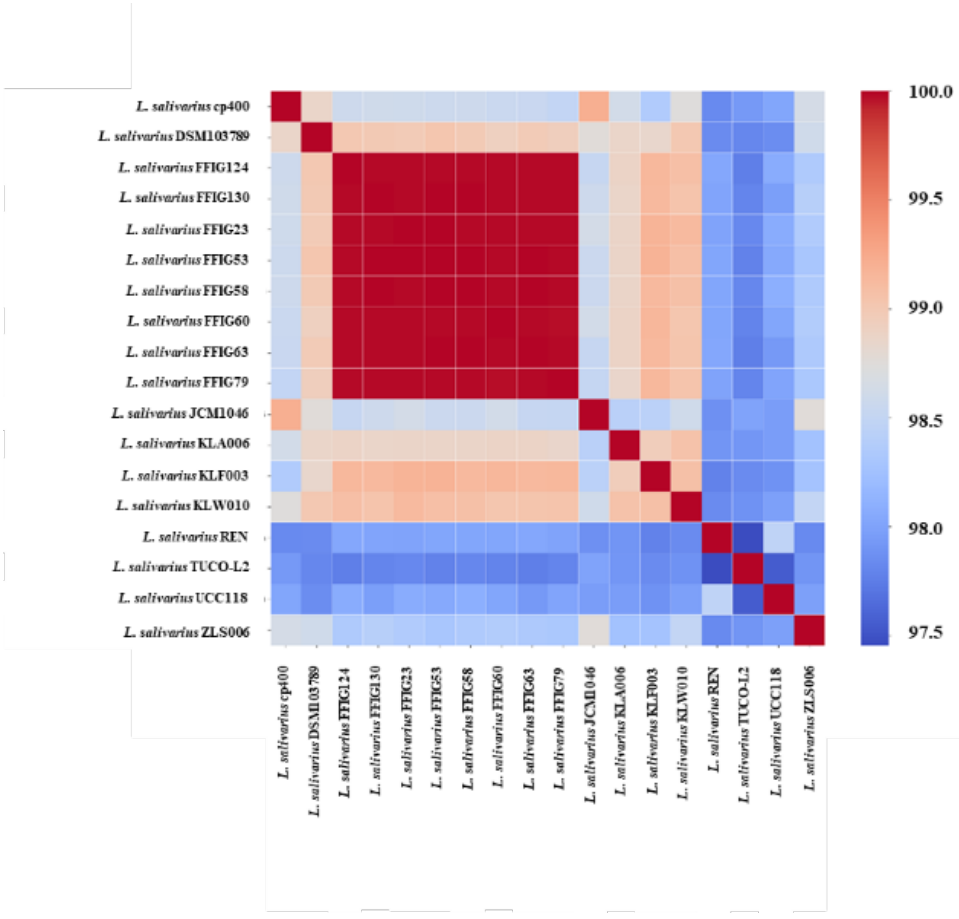


Figure 2-2. Clustering of pair-wise average nucleotide identity (ANI) scores of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs and compared with *L. salivarius* strains with public available complete genomes.

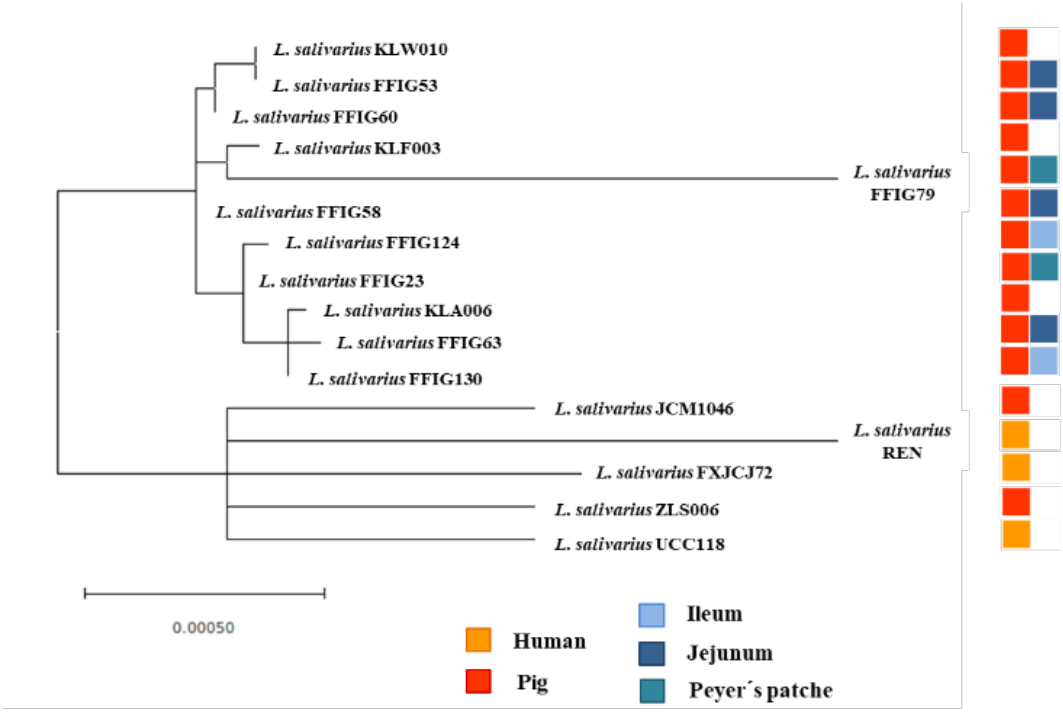


Figure 2-3. Hierarchical clustering of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs. The phylogenetic trees were constructed based on the 16s RNA extracted from the genomes of FFIG strains as well as from *L. salivarius* strains with public available complete genomes.

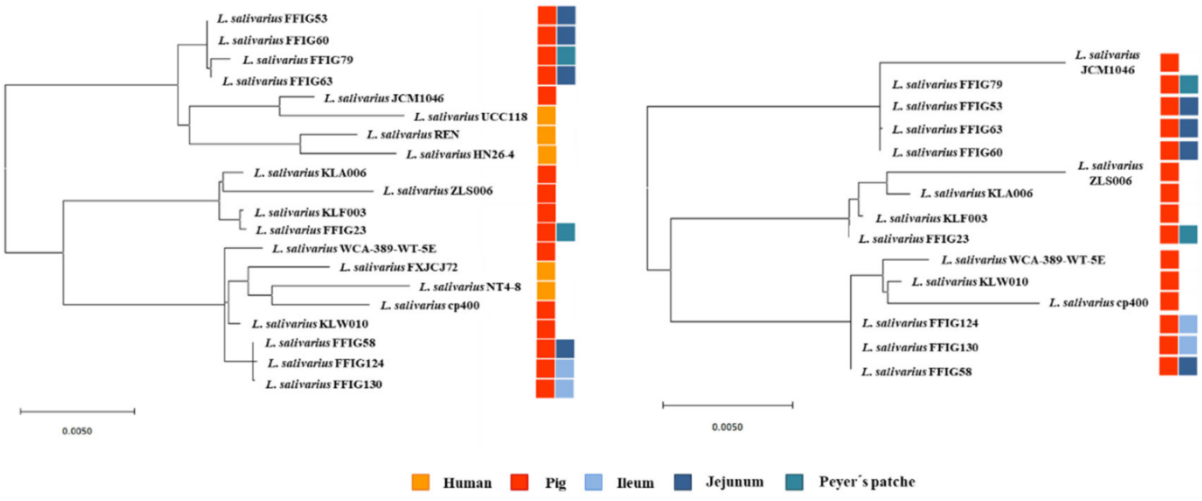


Figure 2-4. Molecular phylogenetic analysis by Maximum Likelihood method of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs. The phylogenetic trees were constructed based on the MLST analysis by using the genes *parB*, *rpsB*, *pheS*, *nrdB*, *groEL*, and *ftsQ* present the genomes of FFIG strains, as well as in *L. salivarius* strains with available public genomes.

Table 2-3. Comparison of the general genome features of sequenced *Ligilactobacillus salivarius* strains with public available genomes. *L. salivarius g*enomes were obtained from NCBI database.

***Ligilactobacillus salivarius* strain**	**Host**	**Sample**	**Genome size (bp)**	**G+C content (%)**	**Protein-coding genes**	**GenBank ID**
A3iob	*Apis mellifera*	Intestine	2,054,490	32.6	1,983	QFAS00000000.1
LPM01	*Homo sapiens*	Milk	1,788,723	33.0	1,717	LT604074.1
CECT 5713	*Homo sapiens*	Milk	1,828,169	32.9	1,884	CP017107.1
HN26-4	*Homo sapiens*	Intestine	1,953,911	32.6	1,811	VSTO00000000.1
FXJCJ7_2	*Homo sapiens*	Intestine	1,891,266	32.8	1,730	VSUK00000000.1
NT4-8	*Homo sapiens*	Intestine	1,910,114	32.7	1,802	VSTK00000000.1
JCM1046	*Sus scrofa*	Intestine	1,836,297	33.1	1,803	CP007646.1
ZLS006	*Sus scrofa*	Intestine	2,177,581	33.2	2,114	CP020858.1
KLA006	*Sus scrofa*	Intestine	2,366,896	32.9	2,276	LXZO00000000.1
KLF003	*Sus scrofa*	Intestine	2,206,918	32.7	2,117	LXZL00000000.1
KLW010	*Sus scrofa*	Intestine	2,389,395	32.7	2,255	LXYX00000000.1
cp400	*Sus scrofa*	Intestine	2,156,840	32.9	1,958	CBVR000000000.1
CICC 23174	*Gallus gallus*	Intestine	1,746,897	33.0	1,606	CP002034.1
DJ-sa-01	*Gallus gallus*	Intestine	1,870,629	33.0	1,719	CP029616.1
UCC118	*Homo sapiens*	Intestine	1,827,111	32.9	1,807	CP000233.1
REN	*Homo sapiens*	Intestine	1,928,516	32.9	1,861	CP011403.1

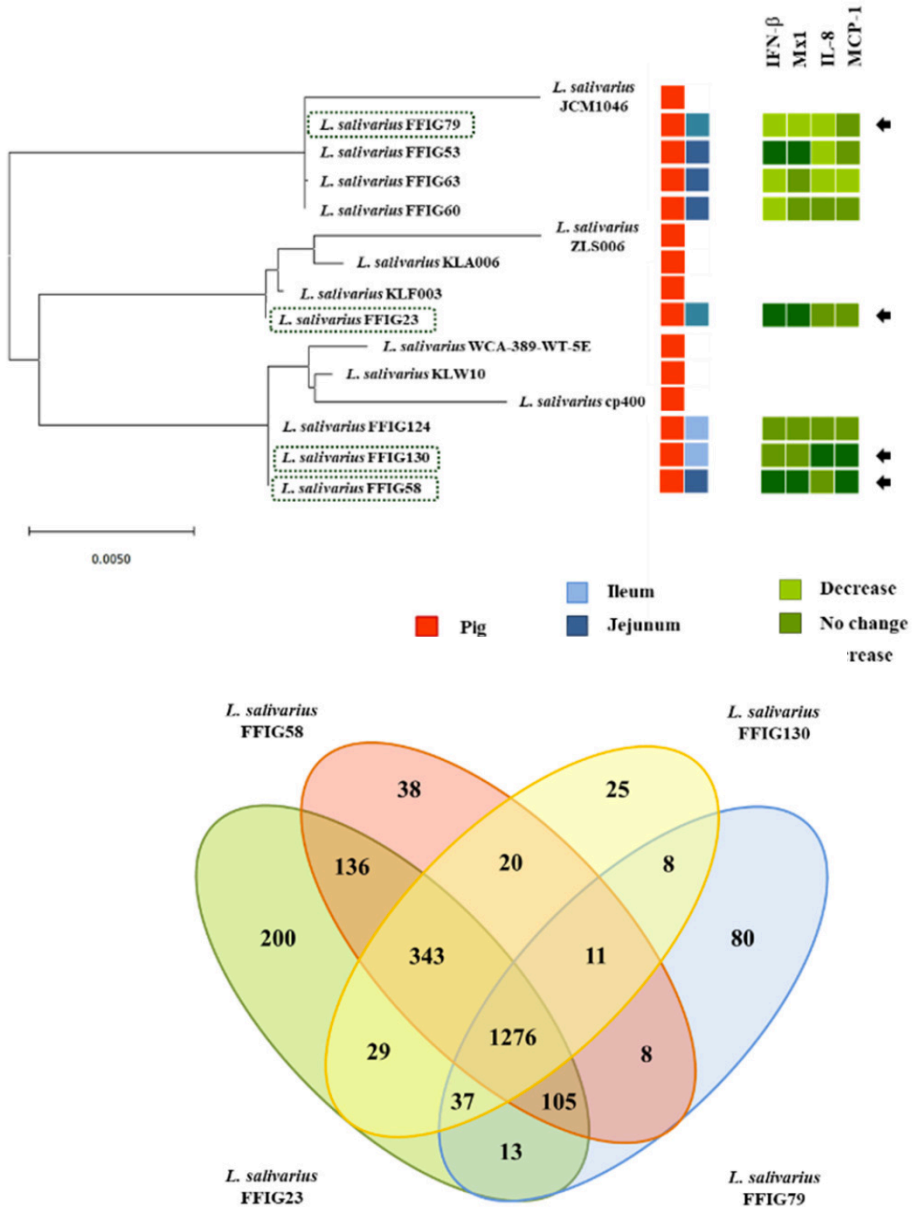


Figure 2-5. Genomic comparison of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs. Four "immune phenotypes" were defined according to the ability of *L. salivarius* FFIG strains of modulating TLRs mediated innate immune responses in porcine intestinal epithelial (PIE) cells. The phylogenetic tree constructed with the MLST analysis of the genes *parB*, *rpsB*, *pheS*, *nrdB*, *groEL*, and f*tsQ* is used to show the strains. *L. salivarius* FFIG23, FFIG58, FFIG79, and FFIG130 were compared. Venn diagram depicts the number of unique genes in each genome and the numbers of genes sheared by the strains.

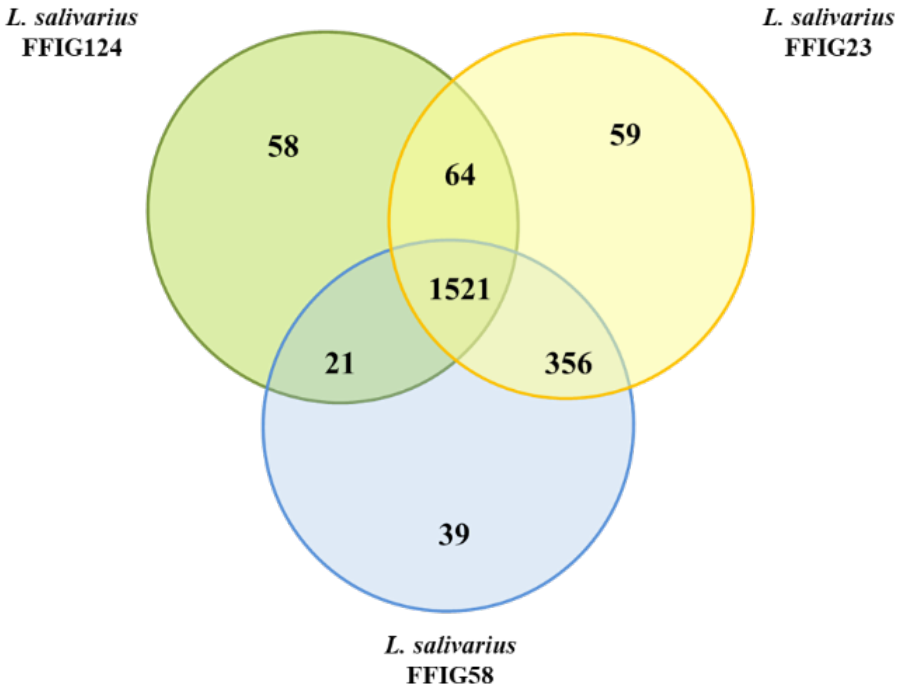


Figure 2-6. Genomic comparison of *Ligilactobacillus salivarius* FFIG58, FFIG23 and FFIG124 isolated from the intestinal mucosa of wakame-fed pigs. Venn diagram depict the number of unique genes in each genome and the numbers of gene sheared by the strains.

2.4. Discussion

In this chapter, PIE cells pre-stimulated by each 116 strains of *L. salivarius* strains were challenged with ETEC or poly(I:C) and the expression of IFN-β, Mx1, IL-8 and MCP-1 were evaluated by RT-PCR. We observed that *L. salivarius* strains isolated from the intestine of wakame-fed pigs modulated the response of PIE cells in a strain-dependent manner. The comparative analysis of IFN-β, Mx1, IL-8, and MCP-1 expression changes clearly showed the different capacities of *L. salivarius* strains in modulating the innate immune responses in the epithelial cells of porcine origin. Our previous studies evaluating the immunomodulatory potential of lactobacilli in PIE cells demonstrated that *Lacticaseibacillus casei* MEP221104 (Basonym: *Lactobacillus casei* MEP221104) improved the expression of IL-1α, IL-6, IL-8, and MCP-1 in ETEC-challenged PIE cells more efficiently than *L. casei* MEP221106. Similarly, *Lacticaseibacillus rhamnosus* MEP221111 (Basonym: *Lactobacillus rhamnosus* MEP221111) was more efficient in the induction of IL-6 and MCP-1 in PIE cells after the activation of TLR4 when compared with *L. rhamnosus* MEP221112[44]. On the other hand, we demonstrated that both *Lactiplantibacillus plantarum* MPL16 (Basonym: *Lactobacillus plantarum* MPL16) and *L. plantarum* CRL1506 were capable of increasing the expression of IFN-β and the antiviral factors Mx2 and RNAseL in poly(I:C)-challenged PIE cells although the MPL16 was more efficient that the CRL1506 strain to induce this effect. In addition, when the two strains were

compared in their ability to modulate the innate antiviral immune response in vivo in a mouse model, *L. plantarum* MPL16 was more efficient than the CRL1506 strain to increase the intestinal levels of IFN-β and IFN-γ, reduce TNF-α and IL-15 and to protect against the TLR3-mediated inflammatory damage[38 45]. In line with our previous investigations, the results obtained in this study indicate that the capacity of the porcine *L. salivarius* strains to differentially modulate the antiviral factors response activated by poly(I:C) stimulation or the production of inflammatory cytokines, induced by ETEC challenge in PIE cells, are strain-dependent characteristics. Even the FFIG strains were isolated from the same niche and under the same environmental pressure (wakame as the main nutritive substrate), they showed different capacities to modulate innate immune responses in PIE cells. This implies that detailed studies of their potentially beneficial properties are necessary to select the strains with the greatest capacity to positively influence the health of the porcine host.

The genes *dapX*, *patA1*, *patA3*, and *patA5* are involved in the acetylation of the peptidoglycan. It was shown that N- and O-acetylation of the cell wall peptidoglycan of gram-positive bacteria are involved in conferring resistance to different types of antimicrobial compounds targeting the cell wall, such as lysozyme, β-lactam antibiotics, endogenous autolysins, and bacteriocins[46 47]. Furthermore, N- and O-acetylation have been shown to differentially modulate the recognition of bacteria by the innate immune system[48]. Importantly, the extent of

the modification of peptidoglycan by acetylation in bacterial cells was shown to vary with species, strain, and even culture conditions[48]. The peptidoglycan O-acetyltransferases *patA* are involved in the O- acetylation of the peptidoglycan. Although the function of *patA* genes has been demonstrated in gram-negative bacteria and streptococci, their function has not been evaluated experimentally in lactobacilli. The results allow us to speculate that differences in the cell wall, in particular, in the molecular structure of peptidoglycan, could explain at least partially the differential immunomodulatory activity observed in the *L. salivarius* strains isolated from the wakame-fed pigs. In line with this hypothesis, we have previously demonstrated that differences in peptidoglycans can confer lactobacilli strains a different ability to interact with the immune system, and consequently, to differentially modulate immune responses [48] [49]. Transcriptomic studies performed in PIE cells and macrophages[49] evaluating the innate immune response triggered by TLR3 activation demonstrated that both *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 were capable to differentially modulate the expression of immune and immune-related genes. When the immunomodulatory effects of viable bacteria were compared with the purified peptidoglycans, only the peptidoglycan from the CRL1505 strain was capable of modulating TLR3-mediated immune response similarly as the viable lactobacilli. Interestingly, the ability of *L. plantarum* CRL1506 to modulate TLR3-mediated innate immune in

PIE cells was exerted at least partially by its lipoteichoic acid and not its peptidoglycan[50].

2.5. Summary

One-hundred and sixteen *L. salivarius* strains isolated from the jejunum, ileum, or ileum Peyer's patches of wakame-fed pigs were evaluated according to their ability to modulate the response of PIE cells to the challenges with TLR3 or TLR4 ligands and expression of IFN-β, Mx1, IL-8 and MCP-1 were evaluated by RT-PCR. Eight strains (*L. salivarius* FFIG23, FFIG53, FFIG58, FFIG60, FFIG63, FFIG79, FFIG124, FFIG130) were selected due to their different immunomodulatory abilities for further studies. Although the FFIG strains were isolated from the same origin and under the same environmental pressure (wakame as the main nutritive substrate), they showed different abilities to modulate innate immune responses in PIE cells.

Phylogenetic trees of eight selected strains were constructed by the Maximum Likelihood method (MLST) analysis with the sequences of 16S rRNA and the genes *parB*, *rpsB*, *pheS*, *nrdB*, *groEL*, and *ftsQ*. The general genomic characteristics of the FFIGs strains were not different from the reported for genomes of *L. salivarius* strains available in public databases. The comparative study of four strains (FFIG58, FFIG23, FFIG130, and FFIG79) belonging to each of the "immune phenotype" groups revealed unique genes related to bacterial cell division, in the biosynthesis of the membrane-forming, the biosynthesis and catabolism of cell-wall peptidoglycan, the biosynthesis of EPS, or integration of membrane proteins, such as lipoproteins. The results allow us to speculate that

differences in the cell wall, in particular, in the molecular structure of peptidoglycan, could explain at least partially the differential immunomodulatory activity observed in the *L. salivarius* strains isolated from the wakame-fed pigs.

Chapter 3

Adhesion capacities of isolated lactobacilli and genomic studies

3.1. Introduction

In Chapter 2, we divided one hundred and sixteen lactobacilli isolates into fourteen groups according to their different immunomodulatory abilities and selected eight strains. In this chapter, the adhesion ability to both human and porcine mucin and PIE cells of the eight strains were evaluated.

Efficient colonization and adhesion to gastrointestinal surfaces is considered important for the probiotic effects of lactobacilli[51] and also a prerequisite of immunomodulatory responses. Lactobacilli perform their adhesion to the intestinal mucosa by interacting with the mucin and recognizing the intestinal epithelium through surface layer proteins[52], moonlighting proteins such as elongation factor Tu (EF-Tu)[53], mucus-binding proteins[54], fimbriae or pili[55 56].

In recent years, comparitive genomic studies have been conducted to find out the critical genetic factors related to different adhesion abilities of selected lactobacilli strains. In this work, we performed comparative genomic studies with the selected FFIG strains focused on genes not only related to bacteria surface like peptidoglycan and EPS but also on specific genes related to adhesion such as the SecA2-SecY2 cluster, mucus-binding proteins, the pilus operon protein and the *Lactobacillus* epithelium adhesin (LEA) protein.

The SecA2/SecY2 secretion system has been associated with the ability of some lactobacilli strains to adhere to mucosal tissues[57 58]. The SecA2/SecY2

genomic cluster encodes the motor protein SecA2, the membrane translocation complex SecY2, the chaperones Asp1-3, and the glycosyltransferases Gtf A and B[59 60]. This system facilitates the glycosylation of srr proteins and the exportation of the glycosylated adhesins that are involved in cell adhesion to the host surface.

The LEA protein was first described in *L. crispatus* ST1, which is an efficient colonizer in chicken intestines and has the capability to adhere to the stratified squamous epithelial cells of the chicken crop[61]. The work described that the LEA protein differed from other sortase-dependent adhesins from lactobacilli, since it harbored no mub repeats, but instead a highly repeated internal region containing Rib/α-like repeats were present in this protein[62]. The complete genome sequence of the ST1 strain allowed the final characterization of the LEA protein of 1898 amino acids, which contains an N-terminal YSIRK signal sequence, the highly repetitive region of Rib/α-like repeats, and a C-terminal LPxTG anchoring motif[62].

Mucin and IECs such as Caco-2 and HT-29 have been used for the *in vitro* evaluation of lactobacilli adhesion[63]. In this chapter, the *in vitro* evaluation of adhesion to mucin was performed by the BIACORE assay that was established by our group[64]. In addition, adhesion experiments were performed with the PIE cell line. We evaluated the adhesion of FFIG strains using this *in vivo* porcine model expecting that the results could be the support for further *in vivo* studies in porcine in the future.

3.2. Materials and Methods

3.2.1. Bacteria culture condition

L. salivarius strains were isolated from the intestine of wakame-fed pigs by using originally developed wakame-based mediums as described previously[14]. The lactobacilli strains and their isolation origin evaluated in this work are listed in Table 3-1. The *L. salivarius* strains isolated from the intestinal tract of wakame-fed pigs were designated as FFIG.

For the experiments of this work, lactobacilli strains were grown in Man–Rogosa–Sharpe (MRS) broth at 37 °C. For the *in vitro* immunomodulatory assays, overnight cultures were harvested by centrifugation, washed three times with sterile phosphate-buffered saline (PBS), counted in a Petroff– Hausser counting chamber, and resuspended in DMEM until use.

3.2.2. PIE Cells

The PIE cell line was originally established at Tohoku University from the intestinal epithelia of an unsuckled neonatal pig, as described previously[37 38]. DMEM medium supplemented with 10% fetal calf serum (FCS), penicillin (100 mg/mL), and streptomycin (100 U/mL) was used for the maintenance of PIE cells. The cells (3.0×10^4 per well) were grown in 12 well type I collagen-coated plates at 37 °C in a humidified atmosphere of 5% CO_2. After three days of culturing period, 1 mL of DMEM containing the different *L. salivarius* strains isolated from

the intestine of wakame-fed pigs (5×10^7 cells/mL) were added to PIE cells monolayers. Cells were further incubated for 48 h at 37 °C, 5% CO_2. PIE cells were washed with fresh medium to eliminate lactobacilli and subsequently stimulated with 10 μg/mL of poly(I:C) (Sigma Aldrich, St. Louis, MI, USA) or enterotoxigenic Escherichia coli (ETEC) for 12 h, to induce the activation of TLR3 and TLR4, respectively. The expressions of IFN-β and Mx1 were evaluated after TLR3 activation, while the expression of IL-8 and MCP-1 were studied after TLR4 stimulation.

3.2.3. Purification of Porcine Intestinal Mucins

Porcine intestinal mucins were used to evaluate the adhesion of FFIG strains[65]. Crude mucus was scraped from porcine small intestinal tissue. Mucus was digested with 0.5 mg/mL of proteinase K (TaKaRa Biotechnology, Shiga, Japan) overnight. After centrifugation (8500 × g, 4 °C, 10 min) and membrane filtration (DISMIC-25, 0.45 μm, Advantec, Tokyo, Japan), the supernatant was purified by gel filtration chromatography with a Toyopearl HW-65F column (90 × 2.6 cm; Tosoh, Tokyo, Japan) using distilled water as the mobile phase. Peptides were detected at 214 nm, and neutral sugar was measured at 490 nm using the phenol-sulfuric acid method. Fractions containing high concentrations of sugars and peptides were collected and concentrated before lyophilization. The purified soluble porcine mucins were used as ligands for the Biacore analysis.

3.2.4. Biacore Assay

Biacore experiments were performed using a Biacore 1000 (GE Healthcare Bio-Sciences K.K., Sheffield, UK) at 25 °C in ab HBS-EP buffer[65]. The immobilization of purified porcine mucins on a CM5 sensor chip (GE Healthcare Bio-Sciences K.K.) was induced by an amine coupling reaction following the manufacturer's instructions. Mucins were dissolved at a concentration of 10 mg/mL in 10 mM sodium acetate buffer (pH 4.0) and immobilized using the reaction between N- hydroxysuccinimide (NHS)-esters and radicals of primary amino groups present in mucins molecules. The sensor chip was equilibrated in an HBS-EP buffer.

Adhesion using the Biacore 1000 is based on the principle of surface plasmon resonance. After washing and lyophilization, bacterial cells were suspended in an HBS-EP buffer (3 mg/mL). The bacterial suspension was injected at a flow rate of 3 μL/min for 5 min, the sensor chip was washed with HBS-EP buffer to remove unbound analyte; and regenerated eluting with 1 M guanidine hydrochloride (GHCl) solution at a flow rate of 3 μL/min for 2 min. The resonance units (RU) were measured for 200 s after the cessation of sample addition. A response of 1 RU represents 1 pg/mm^2 protein adhering with an increased concentration of analyte bound to the sensor chip surface.

3.2.5. Adhesion to PIE Cells

The adhesion of lactobacilli to PIE cells was performed by the micro-plate method using fluorescent bacteria[66]. Cultured lactobacilli were washed with PBS three times (6000 rpm, 10 min). Pellet was resuspended in 1 mL PBS, and 1 mM of carboxyfluorescein diacetate (CFDA) was added for the fluorescent reaction at 37 °C for 1 h. Then, bacteria were washed with PBS three times (6000 rpm, 10 min) to remove CFDA on the microbial surface. Fluorescent bacteria were counted by hemocytometer.

PIE cells were seeded at 5000 cells/well in Type I collagen-coated 96 well cell culture plate (Nippi Incorporated, Tokyo) for 3 days. Cultured fluorescent lactobacilli were added to PIE cells at 100 MOI and co-cultured for 48 h. After incubation, non-adherent bacteria were washed out with PBS. After lysis with 0.1 N NaOH, fluorescence was evaluated by 2030 Multilabel Reader (Perkin Elmer, Fukuoka, Japan).

3.2.6. Scanning Electron Microscopy (SEM) Analysis

Lactobacilli were washed once and diluted 2 times with PBS. The suspension with bacteria was dropped on the polycarbonate membrane (ADVANTEC) and filtered with vacuum filtration (Miripore). The membrane with lactobacilli on the surface was immersed in 2% (v/v) glutaraldehyde solution. After 1 h, the membrane was immersed in 50, 60, 70, 80, 90, and 99% ethanol for 20 min at a time to remove water. The membrane was immersed in t-butyl

alcohol, lyophilized, and treated with platinum palladium. SEM observation was performed in a HITACHI microscope.

3.2.7. Bioinformatic Analysis

The genome sequences of *L. salivarius* were downloaded from the GenBank database (https://www.ncbi.nlm.nih.gov/genome/1207). The average nucleotide identity (ANI) was calculated using the Enveomics collection web[67]. Phylogenetic trees were constructed to examine the relationships between the different microorganisms. The gene sequences were downloaded from the GenBank databases. The MUSCLE aligner[68] available in the MEGAX (v.10.0.4)[69] software, was employed to align the gene sequences of all microbes before the construction of the phylogenetic tree according to the Neighbor-Joining (NJ) distance algorithm [70] [71] embedded in the MEGAX software as well. Heat-map figures were constructed using tools for plotting data[72] in R scripts. Pangenome analysis was conducted with Roary (v.3.6.0)[73] using the Prokka annotation (v.1.12)[74]. De Venn diagrams were generated with InteractiVenn[75].

“About Biacore”

Biacore is a system for real-time biomolecular interaction analysis. It monitors the formation and dissociation of biomolecular complexes on a sensor surface as the interaction occurs. By covalently attaching one molecule (ligand) to the surface, the interaction of another molecule in solution (analyte) with the ligand is followed. Measurements are made under conditions of continuous flow. For the majority of applications, the biospecific surface can be regenerated and reused for an extended series of analyses.

The measurement in Biacore is performed using surface plasmon resonance (SPR). The SPR is a non-invasive optical measuring technique which measures the mass concentration of biomolecules in close proximity to a specially prepared surface (Fig. 3-1). The technique does not require any labeling of the interacting components. The response is essentially independent of the nature of the biomolecule, so that all steps in an interaction analysis may be followed with the same detection technique.

In a Biacore adhesion aasay, the following steps are proformed. A sensorgram is a plot of response against time (Fig. 3-1), showing the progress of the interaction. After the ligand immobilized on a sensor chip, the solution of analyte can be injected to the selected flow cell. When the injection of analyte stops, the buffer from the system will start to flow. The adhesion value should be observed in this step (Fig. 3-2). Finally, by an injection of acidic (glycine-HCl

buffer or dilute HCl), basic (NaOH) or surfactant solutions, the bound analyte should be removed from the sensor chip surface without destroying the ligand activity. The process here is called regeneration. The number of times a sensor surface can be regenerated depends on the nature of the attached ligand, but is usually greater than 100. Time spent on the adhesion assay would be about 20 min one time. The amount of ligand and analyte is not constant in every test. For most proteins, concentrations of 10-50 μg/mL are sufficient, and higher concentrations simply consume more ligand without significantly improving the results. Lower concentrations may be used in favorable cases. The analyte concentration is around 1-3 mg/mL in this study.

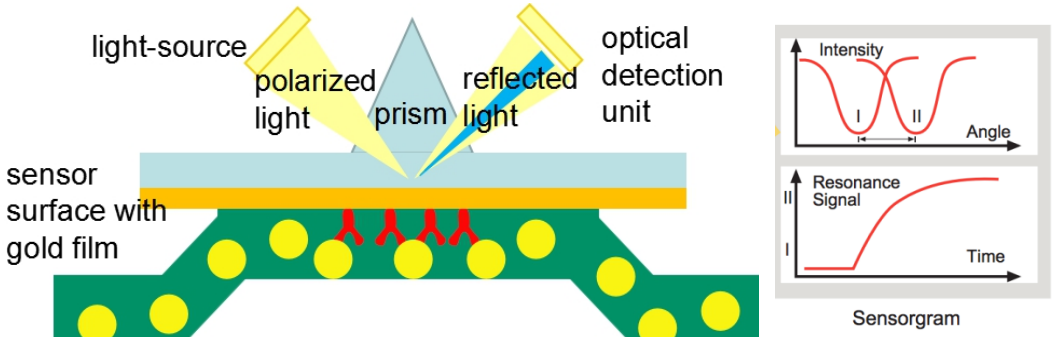


Figure 3-1. A ligand-analyte interaction of Biacore analysis using surface plasmon resonance.

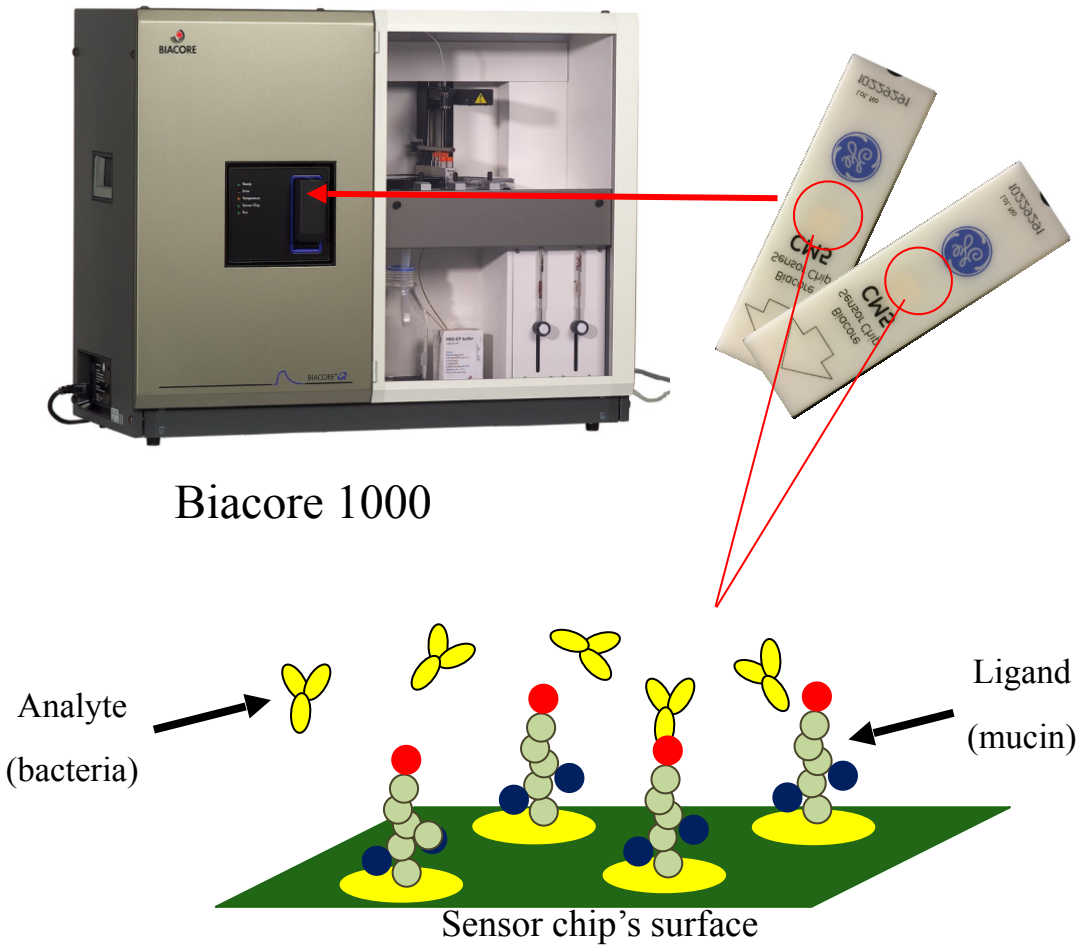


Figure 3-2. Outline of Biacore apparatus

Table 3-1. Species, strains and origins of the bacteria used in this chapter.

Species	**Strain**	**Origin (porcine)**
Ligilactobacillus salivarius	FFIG23	Ileum peyer's patch
Ligilactobacillus salivarius	FFIG53	Jejunum
Ligilactobacillus salivarius	FFIG58	Jejunum
Ligilactobacillus salivarius	FFIG60	Jejunum
Ligilactobacillus salivarius	FFIG63	Jejunum
Ligilactobacillus salivarius	FFIG79	Ileum peyer's patch
Ligilactobacillus salivarius	FFIG124	Ileum
Ligilactobacillus salivarius	FFIG130	Ileum

3.3. Results

3.3.1. Preparation of soluble human and porcine mucin

Both human and porcine mucus tissues were purified with Proteinase K to digest these proteins and the core protein structure of mucin. After gel filtration chromatography, the fractions containing high concentrations of sugars and peptides were collected and lyophilized (Fig. 3-3). We collected fraction number 61~111 from porcine mucin and fraction number 31~79 from human mucin. Compared to human colonic mucin, porcine intestinal mucin contains more peptide and neutral sugar. Soluble human colonic mucin (sHCM) and soluble porcine intestine mucin (sPIM) were used as ligand in BIACORE assay.

3.3.2. Adhesion capacity to mucin and PIE cells

All the studied strains had the ability to adhere to porcine mucins, a fact that was expected considering the origin of the FFIG strains. *L. salivarius* FFIG23, FFIG53, FFIG58, FFIG124, and FFIG130 had a modest capacity to adhere to porcine mucins as shown by the resonance units that had values below 6. The FFIG60 and FFIG63 strains had a moderate ability to adhere to porcine mucins (resonance units between 6 and 8), while the FFIG79 had a remarkable capacity to bind and adhere to porcine mucins (resonance units above 8). Almost all *L. salivarius* strains isolated from wakame-fed porcine adhered more to sPIM than sHCM (Fig. 3-4). FFIG79, FFIG63 and FFIG60 showed significantly better

adhesion ability to porcine mucin than human mucin. *L. salivarius* FFIG23, FFIG53, FFIG58, FFIG124 and FFIG130 showed weak ability to adhere to porcine mucin by the resonance units below 6. FFIG60, FFIG63 showed a moderate ability to porcine mucin by the RU value between 6 and 8. FFIG79 had a remarkable capacity to bind and adhere to porcine mucins (RU value was above 8) (Fig. 3-4).

We also observed a strain-dependent effect when the adhesion of lactobacilli to PIE cells was evaluated (Fig. 3-5). *L. salivarius* FFIG23, FFIG53, FFIG60, and FFIG79 could not adhere to PIE cells as shown by the fluorescence units that were not different from control cells. The FFIG124 and FFIG130 strains had a moderate ability to adhere to PIE cells, while the FFIG58 and FFIG63 had a remarkable capacity to bind and adhere to PIE cells, being the adhesion of the FFIG58 strain the most notorious among all the strains evaluated. Taking into consideration the outstanding differences between the FFIG58 and other strains, such as the FFIG79 in their capacity to adhere to PIE cells, we performed an SEM analysis of these two *L. salivarius* strains to evaluate the presence of bacterial structures, such as fimbriae, that may explain their different behavior (Fig. 3-5). No evident differences were found in the SEM analysis when the *L. salivarius* FFIG58 and FFIG79 were compared. These results indicate that at least under these experimental conditions, the FFIG58 strain does not present evident surface

structures that may be associated with its greater ability to adhere to porcine epithelial cells.

3.3.3. Comparative genomic analysis of "adhesion phenotypes"

We next carried out comparative genomic studies to characterize the *L. salivarius* FFIG strains with different adhesion capacities. Thus, we defined "adhesion phenotypes" according to the ability of the FFIG strains to adhere to porcine mucins or to PIE cells (Fig. 3-6). The strain FFIG58 that had a high capacity to adhere to PIE cells and low ability to adhere to mucins, and the strain FFIG79 with the exact opposite behavior were considered as different "adhesion phenotypes". In addition, the strain FFIG63 with high capacity to adhere to PIE cells and moderate ability to adhere to mucins, and the strain FFIG23 with the moderate capacity to adhere to porcine mucins and low adhesion to PIE cells were considered as two "adhesion phenotypes" groups (Fig. 3-6).

The comparative study of the strains FFIG58, FFIG23, FFIG63, and FFIG79 revealed a coregenome of 1,204 genes (Fig. 3-6). The strains with the ability to adhere to PIE cells, *L. salivarius* FFIG58, and FFIG63, had 49 and 47 unique genes, respectively. In addition, the FFIG58 and FFIG63 strains sheared nine genes that were not found in FFIG23 or FFIG79. Among the unique genes of the FFIG58 strain, we found a fap1-like adhesin (*fap1*), while in the genes shared by FFIG58 and FFIG63, we were not able to identify genes potentially involved

in adhesion to mucins or intestinal epithelial cells. On the other hand, among the unique genes of *L. salivarius* FFIG63, we identified a glycosyltransferase EpsJ (*epsJ*). The strain *L. salivarius* FFIG23 and the FFIG58 sheared 183 genes, including a type 4 prepilin-like proteins leader peptide-processing enzyme or membrane prepilin peptidase (*comC*). In addition, the highly porcine mucin-adhesive strain *L. salivarius* FFIG79 had 64 unique genes, including a putative agglutinin receptor (*ssp5*) that binds sialic acid residues of salivary agglutinin in a calcium-dependent reaction. Among the genes sheared by FFIG23 and FFIG79, we found a lipoteichoic acid synthase 1 (*ltaS1*).

We also performed a comparative genomic study of the strains FFIG58 and FFIG79 (with high adhesion to PIE cells and porcine mucin, respectively) with the less adherent strain FFIG60 (Fig. 3-7). The comparison of these three strains revealed a coregenome of 1,362 genes. The strains FFIG58, FFIG79, and FFIG60 had 122, 69, and 53 genes, respectively. Among the unique genes in the FFIG60 strain, we found a glycosyltransferase EpsH (*epsH*) and the lipoteichoic acid synthase 1 (*ltaS1*). The FFIG60 and FFIG79 strains sheared the gene for a glycosyltransferase EpsJ (*epsJ*). Of note, the FFIG60 and FFIG58 sheared 385 genes, and among them, we detected a sugar transferase EpsL (*epsL*), a glycosyltransferase EpsD (*epsD*), and the Fap1-like adhesin (*fap1*).

3.3.4. Analysis of adhesion factors in *L. salivarius* isolated from the intestinal tract of wakame-fed pigs

3.3.4.1 The SecA2/SecY2 secretion system

It was reported that *L. salivarius* strains isolated from pigs and chickens possess the SecA2-SecY2 cluster, while this system was not detected the human *L. salivarius* isolates[42]. In agreement with this previous finding, we were not able to detect the SecA2-SecY2 cluster in the human-related *L. salivarius* strains UCC118 and REN, which we used as references in this work. Moreover, the SecA2-SecY2 cluster was found in all the genomes of *L. salivarius* strains isolated from the intestine of wakame-fed pigs (Fig. 3-8). Although all the FFIG strains contained the genes of the SecA2-SecY2 system, our results evaluating the phylogenetic clustering, based on the nucleotide sequences of the conserved genes *secA2*, *secY2*, *asp1*, *asp2*, *asp3*, *gtfA*, and *gtfB*, showed some degree of divergence. The FFIG23, FFIG53, and FFIG58 clustered separately from the other *L. salivarius* strains isolated from the intestine of wakame-fed pigs and together with the porcine strain KLW002. No differences were detected between the FFIG60, FFIG63, FFIG79, FFIG124, and FFIG130. Of note, all the FFIG strain clustered separately from the porcine strains JCM1046 and ZLS006 and the chicken strain DJ-sa-01 (Fig. 3-8).

We further analyzed the srr proteins located within the SecA2-SecY2 system cluster in all the FFIG strains to detect potential differences between them

(Fig. 3-9). The srr protein found in the genome of *L. salivarius* FFIG58 (detected as a fap1-like adhesin in Fig. 3-6) was identical to the srr protein found in the KLW002 genome and slightly different from the srr proteins located in the SecA2-SecY2 system cluster of the other FFIG strains. However, the only difference found between the distinct srr proteins among the FFIG strains was in their length, while the similarity between them was 97-99%. Furthermore, the phylogenetic clustering, based on their nucleotide sequences, revealed no significant divergence (Fig. 3-9). Interestingly, the FFIG strains clustered together with the porcine strain JCM1046 when the srr proteins were compared despite the large difference in the size in their genes. In contrast, evident differences were found when the srr proteins were compared with the porcine strain ZLS006 and the chicken strain DJ-sa-01 (Fig. 3-9).

3.3.4.2 Mucus-binding proteins

We searched for MucBPs genes in the genomes of *L. salivarius* strains isolated from the intestine of wakame-fed pigs focusing our attention in the "adhesion phenotypes" groups, which included the FFIG23, FFIG58, FFIG63, and FFIG79 strains. In addition, we searched for MucBPs genes in the genomes of the strains UCC118, REN, DJ-sa-01, CICC23174, JCM1046, and ZLS006 for comparison (Fig. 3-10).

As described previously[42], a common MucBP (designated here as MucBP1) was found in all the *L. salivarius* strains, independently of the host origin. The MucBP WP_087118522.1 (designated here as MucBP2) was found in the genomes of the UCC118, DJ-sa-01, JCM1046, and ZLS006, as well as in the strains FFIG23, FFIG58, and FFIG63, but not in the genome of FFIG79. In addition, a MucBP WP_179219866.1 (designated here as MucBP3) was sheared by the REN strain and the *L. salivarius* FFIG strains, while the protein WP_172824493.1 detected in the genomes of UCC118 and JCM1046 were not found in the FFIG strains (Fig. 3-10). MucBP1, was also found in the genomes of the other FFIG strains. MucBP2 was detected in the genomes of FFIG60 and FFIG124, but not in FFIG53 and FFIG130 strains, while MucBP3 was present in FFIG130, but not in FFIG53, FFIG60, and FFIG124 (data are not shown). Our analysis was not able to identify a clear association between the presence of MucBPs and the different adhesion capabilities of the FFIG strains.

3.3.4.3 Pilus operon proteins

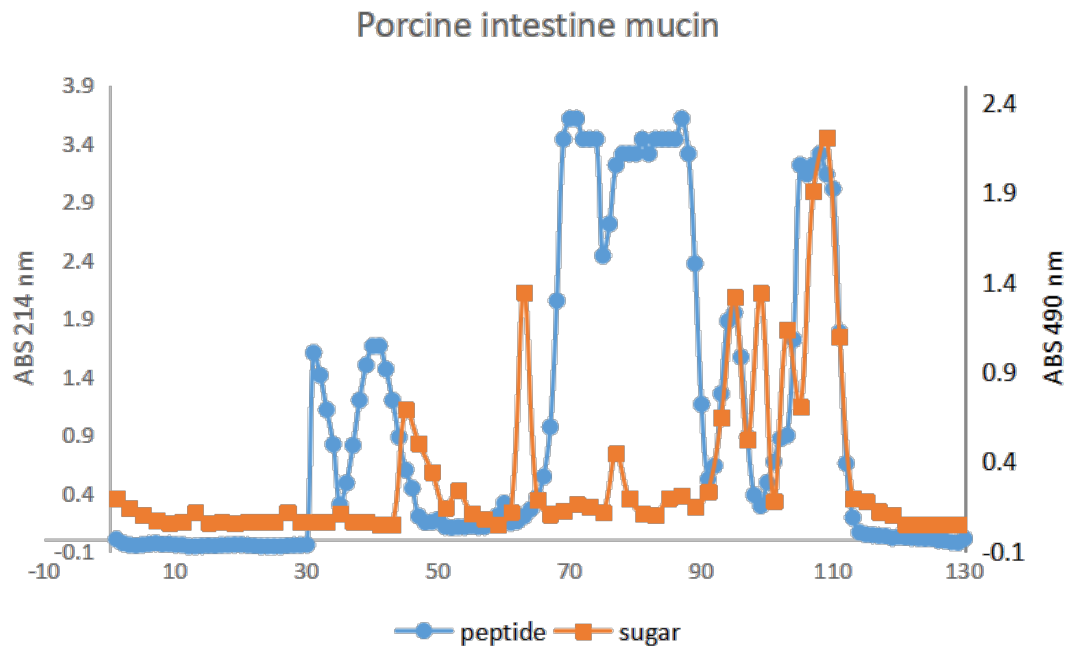
Pili are other bacterial structures involved in the intestinal colonization of lactobacilli, which have also been associated with probiotic effects[76]. The pilus is comprised of two to three types of pilin subunits, each of them with its own distinct location and role in the molecular structure. In its biosynthesis, a pilus-specific sortase type C catalyzes the head-to-tail assembly of the pilins into the

final polymerized form[77]. Despite the fact that the presence of pilus in probiotic strains belonging to the species *L. salivarius* has been scarcely evaluated, this bacterial structure is predicted to exist in this species based on genomics analyses. In fact, genomic studies performed by Harris *et al.*[43] showed that among 43 *L. salivarius* genomes, only five contained the genes for an extra sortase A, a sortase C, and putative pilin subunits. These strains are *L. salivarius* JCM1047, DSM20555, ATCC11741, gul1, and gul2. We also searched for other *L. salivarius* strains with pili operon in the NCBI genome bank and found this cluster only in the genome of *L. salivarius* A3iob originally isolated from the bee intestine[78]. Moreover, the comparative analysis of the pili operon sequences in these six strains showed that they harbor only three different pili operons (data are not shown). Then, we used the pili operons of JCM1047, DSM20555, and the A3iob for the genomic comparisons with the *L. salivarius* FFIG23, FFIG58, FFIG63, and FFIG79 (Fig.3-8). No genes for an extra sortase A, a sortase C, or pilin subunits were found in these four strains (Fig. 3-8) or in the other *L. salivarius* strains isolated from the intestine of wakame-fed pigs (data are not shown).

3.3.4.4 *Lactobacillus* epithelium adhesin (LEA)

We also investigated the presence of the Lactobacillus epithelium adhesin (LEA) family of proteins in the genomes of *L. salivarius* strains. The genomic analysis of the FFIG strains revealed the presence of a similar protein annotated as

LEA family epithelial adhesin. The LEA family adhesin from *L. salivarius* FFIG58 is a protein of 1578 amino acids, and contains an N-terminal YSIRK signal sequence, a C-terminal LPxTG anchoring motif, and 11 Rib/α-like repeats (Fig. 3-11). The LEA proteins in all the *L. salivarius* strains isolated from the intestine of wakame-fed pigs were identical (data are not shown), and they were also similar to the LEA proteins found in the genome of *L. salivarius* strains of porcine origin. In fact, the LEA family adhesin from the porcine strain *L. salivarius* ZLS006 has the YSIRK and LPxTG motifs together with 12 Rib/α-like repeats (Fig. 3-11). The LEA family adhesins from FFIG58 and ZLS006 were 90.61% identical.



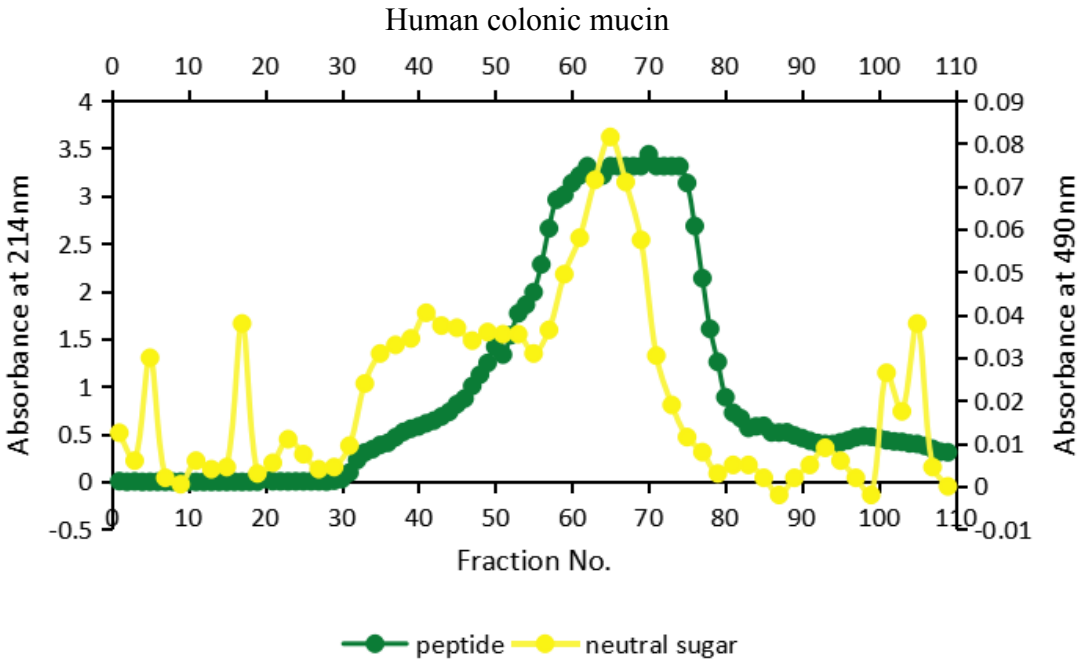


Figure 3-3. The purification of soluble porcine intestine mucin and soluble human colonic mucin (type A) by gel filtration chromatography was detected by UV absorption at 214 nm (peptide) and phenol-sulfuric acid method at 492 nm (neutral sugar).

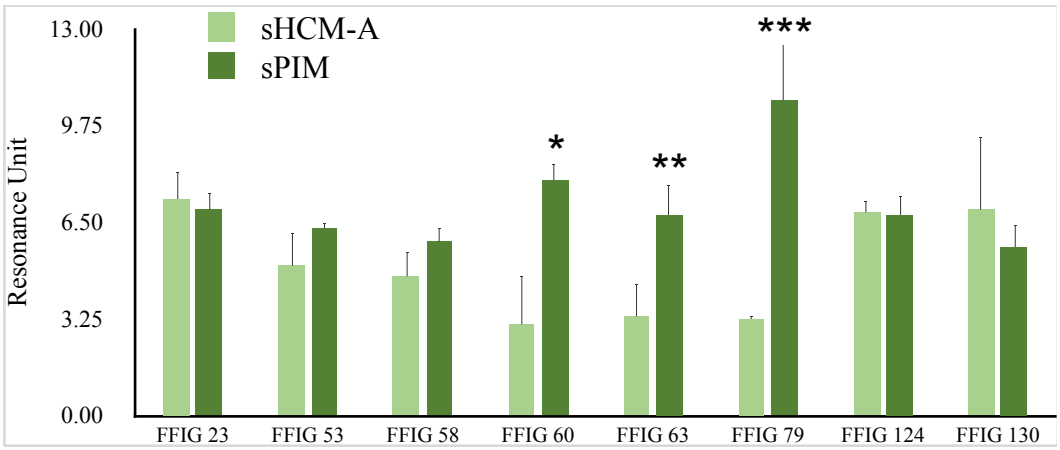


Figure 3-4. Adhesion abilities of *Ligilactobacillus salivarius* strains to sPIM and sHCM using Biacore analysis. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to the adhersion to sPIM.(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Against sPIM).

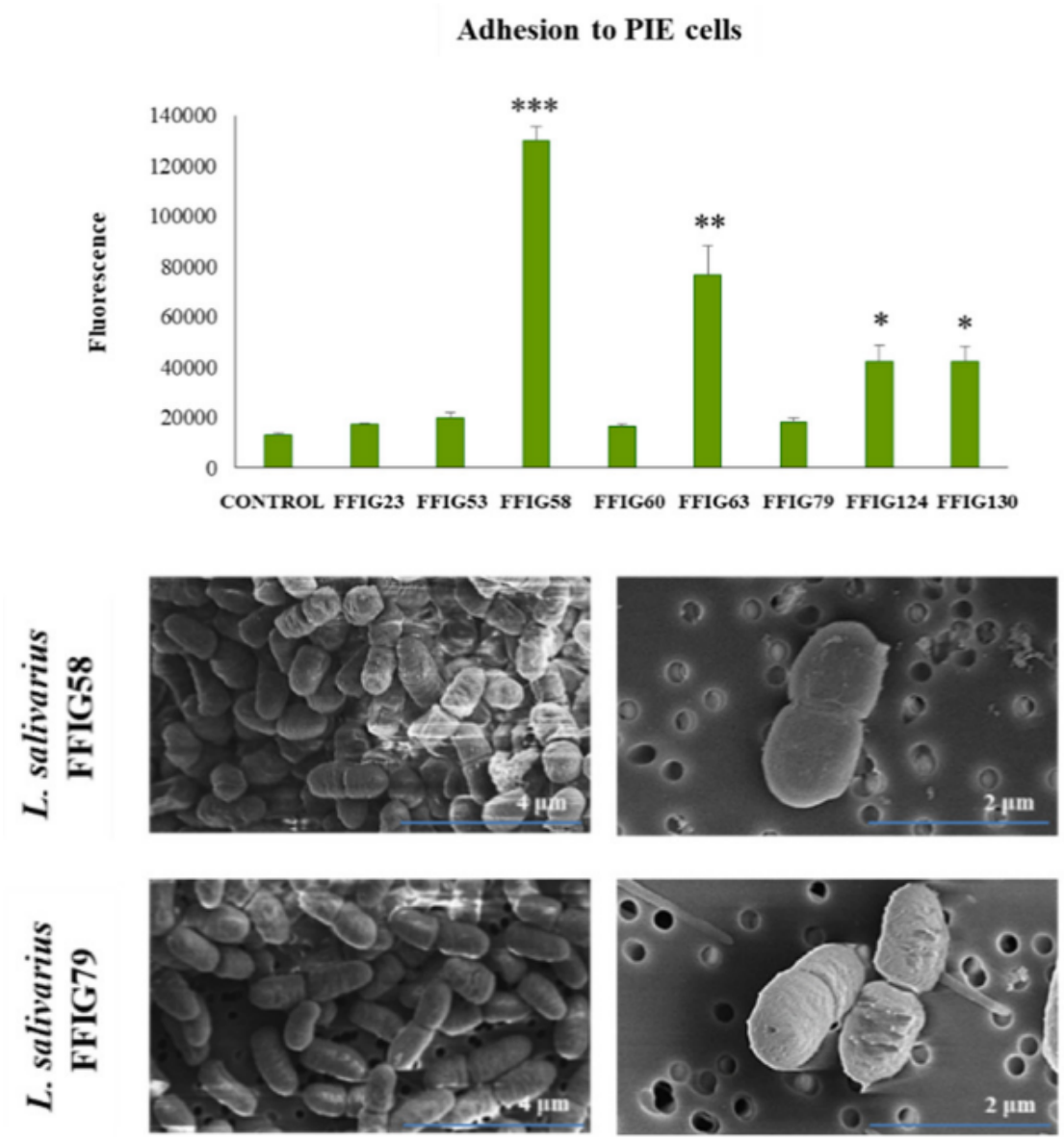


Figure 3-5. Adhesion of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs to porcine intestinal epithelial (PIE) cells. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to the control PIE cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Against Control). Scanning electron microscope (SEM) analysis of *L. salivarius* FFIG58 and *L. salivarius* FFIG79.

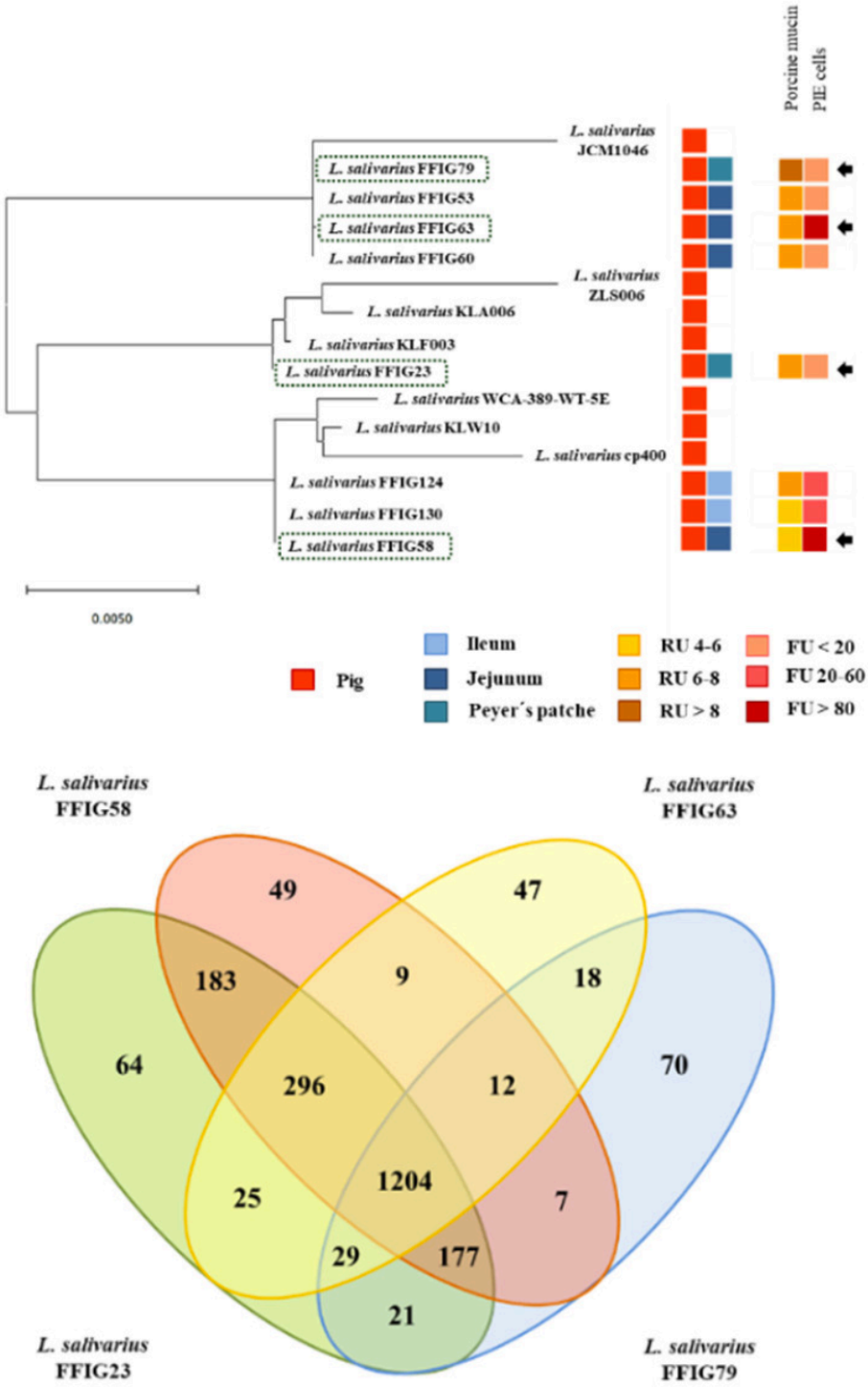


Figure 3-6. Genomic comparison of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs. Four "adhesion phenotypes" were defined according to the ability of *L. salivarius* FFIG strains to adhere to porcine mucins, as well as to porcine intestinal epithelial (PIE) cells. The phylogenetic tree constructed with the MLST analysis of the genes *parB*, *rpsB*, *pheS*, *nrdB*, *groEL*, and *ftsQ* is used to show the strains. *L. salivarius* FFIG23, FFIG58, FFIG63, and FFIG79 were compared. Venn diagram depicts the number of unique genes in each genome and the numbers of genes sheared by the strains.

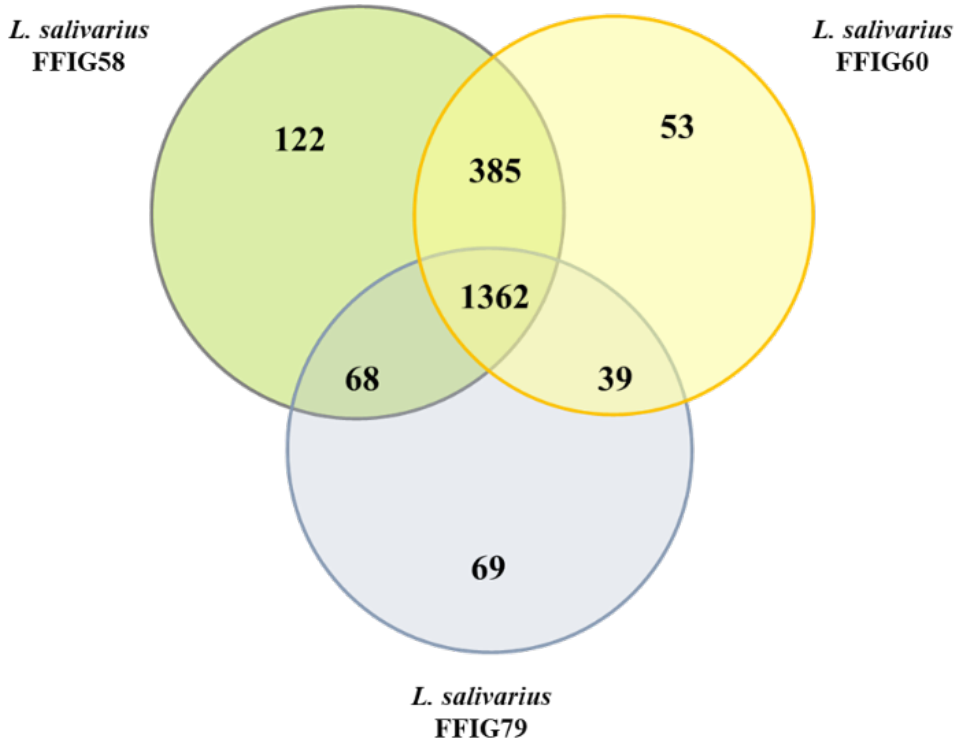


Figure 3-7. Genomic comparison of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs. Three "adhesion phenotypes" defined according to the ability of *L. salivarius* FFIG strains to adhere to porcine and human mucins and to porcine intestinal epithelial (PIE) cells were compared. *L. salivarius* FFIG58, FFIG60 and FFIG79. Venn diagram depict the number of unique genes in each genome and the numbers of gene sheared by the strains.

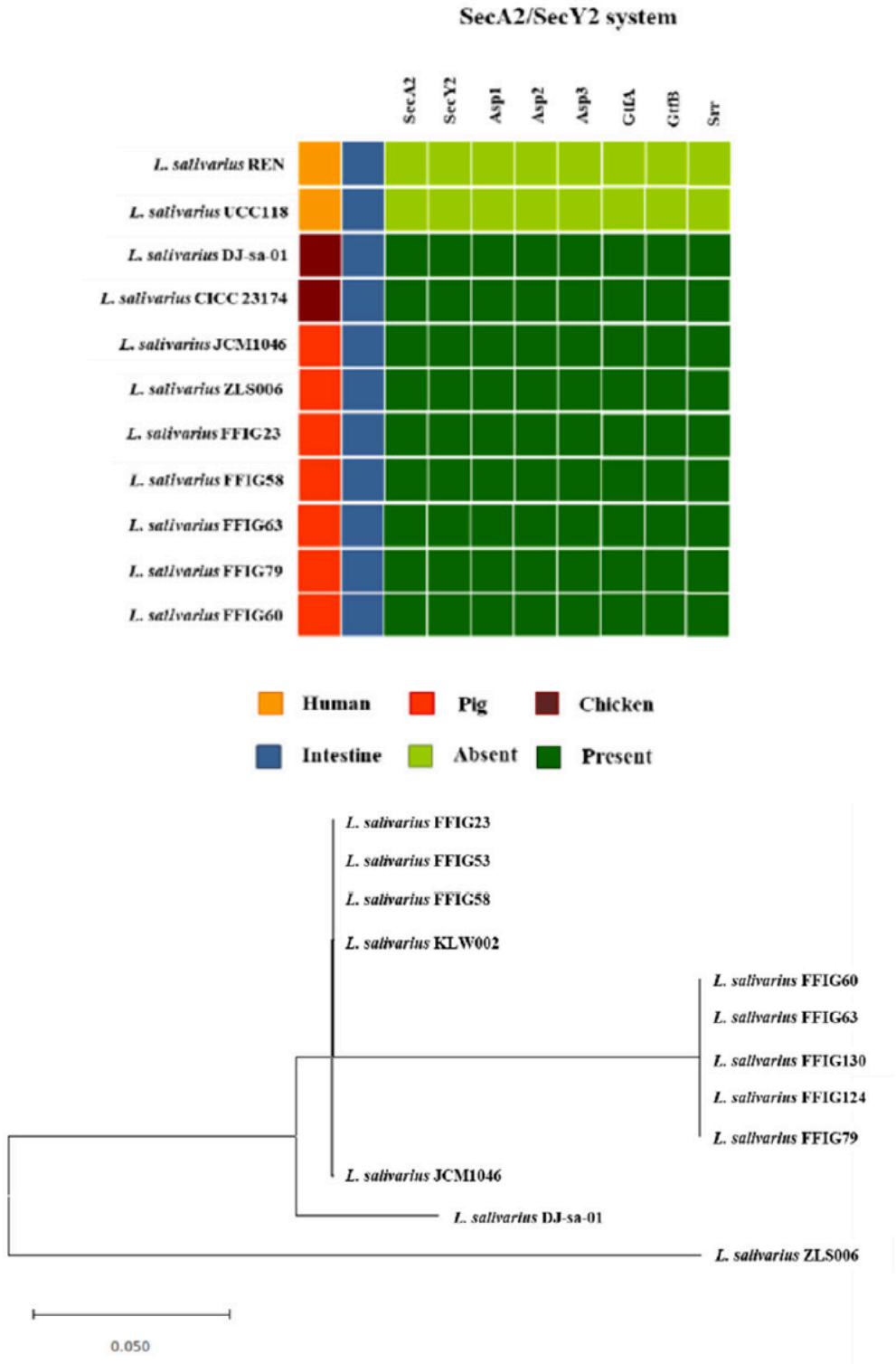


Figure 3-8. Genomic comparison of the SeA2-SecY2 accessory secretion system from *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs and probiotic *L. salivarius* strains isolated from the intestinal tract of humans, pigs, and chickens. The phylogenetic tree was constructed by using the sequences of the genes *secA2, secY2*, *asp1*, *asp2*, *asp3*, *gtfA*, and *gtfB* sheared by the strains of animal origin.

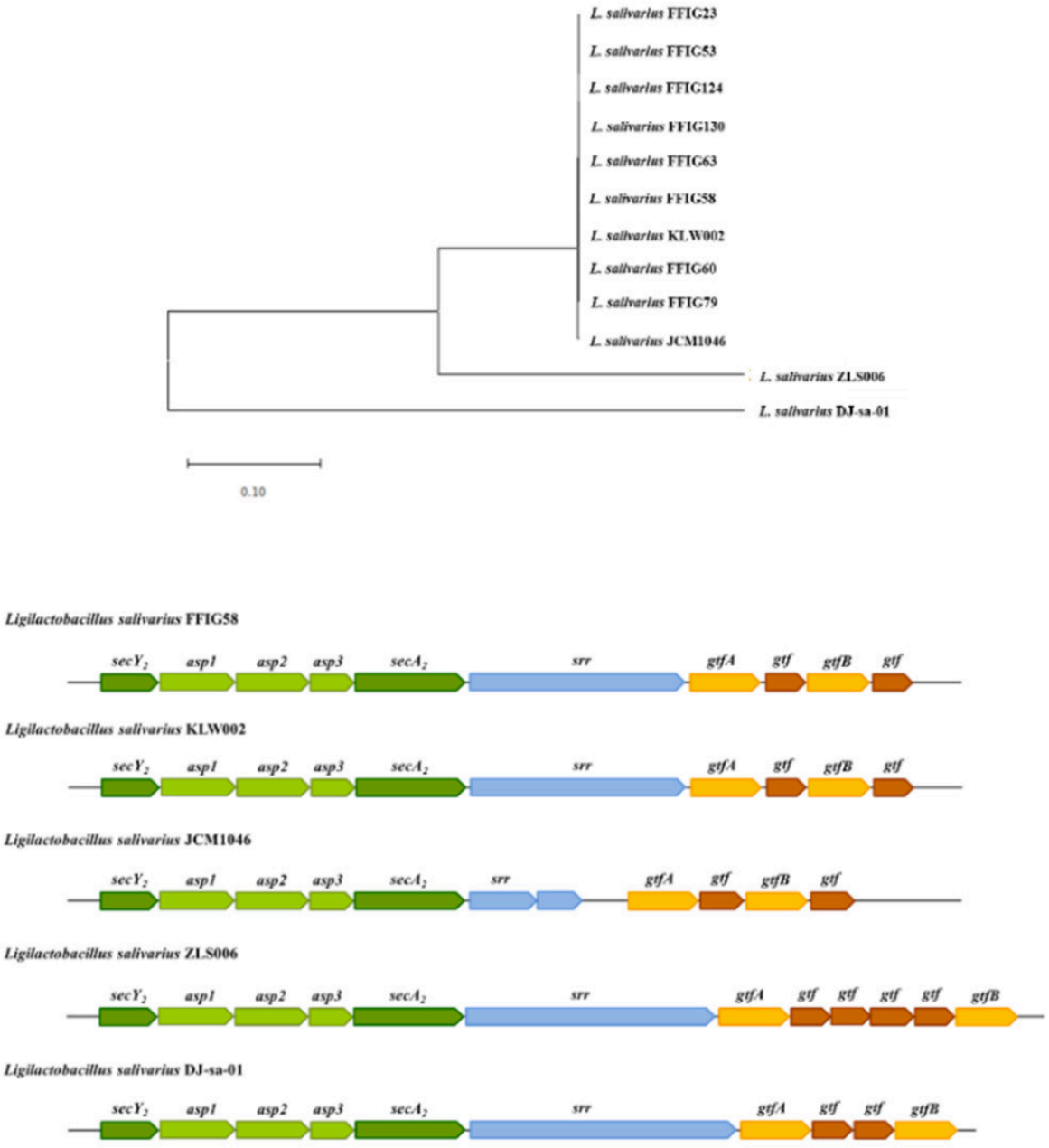


Figure 3-9. Genomic comparison of the SeA2-SecY2 accessory secretion system from *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs and probiotic *L. salivarius* strains isolated from the intestinal tract of pigs, and chickens. The phylogenetic tree was constructed by using the sequences of the *srr* genes belonging to the SeA2-SecY2 cluster. Gene organization within the SeA2-SecY2 accessory secretion system for selected strains is shown. Conserved glycosyltransferases are shown in yellow, while non-conserved glycosyltransferases are shown in brown.

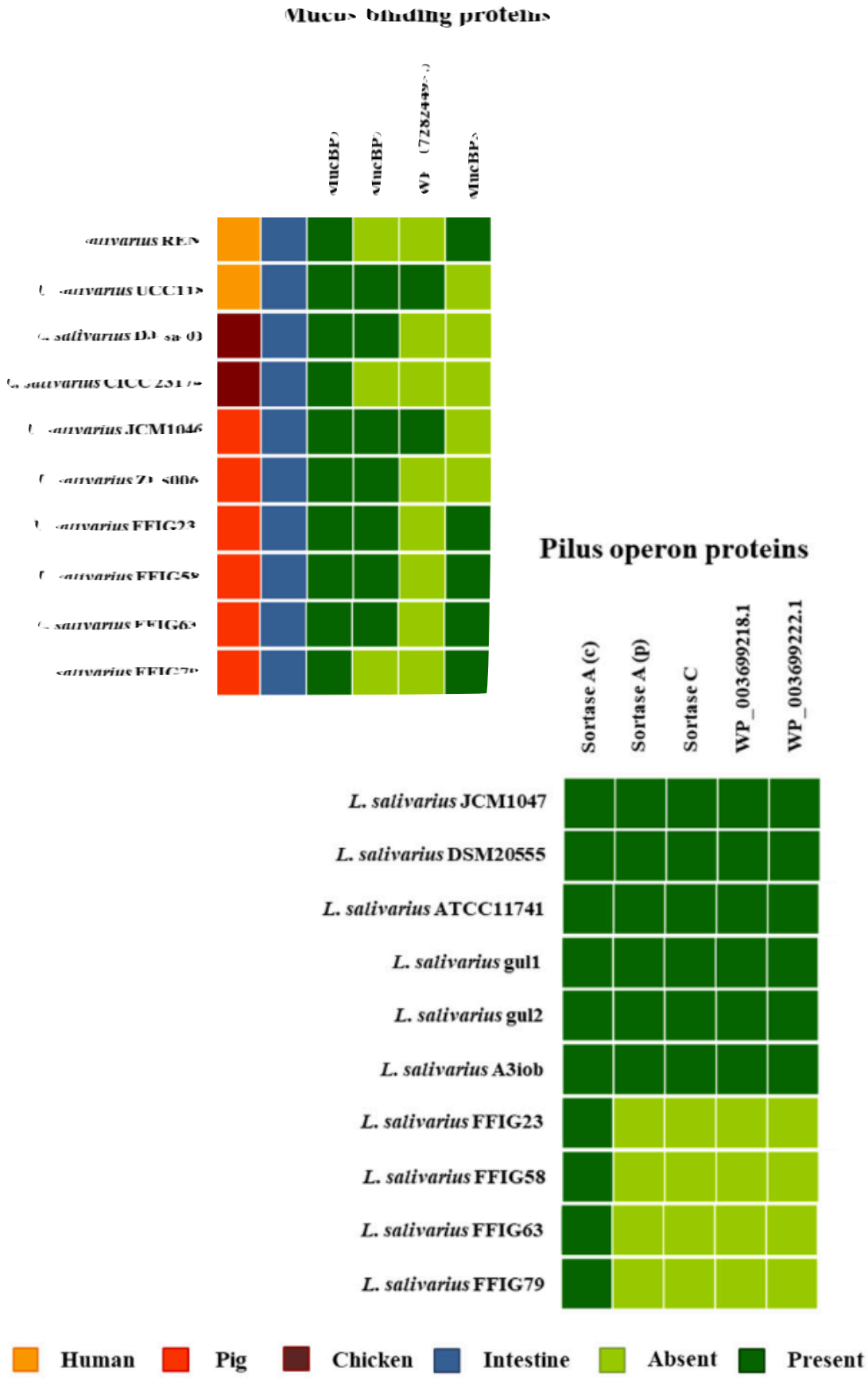


Figure 3-10. Genomic comparison of the mucus binding proteins and the pilus operon from *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs with *L. salivarius* strains with available public genomes. Mucus binding proteins were compared with probiotic *L. salivarius* strains isolated from the intestinal tract of humans, pigs, and chickens. Gene of proteins associated with pilus operon were compared to the six *L. salivarius* strains that were predicted to harbor a pilus operon by genomic analysis.

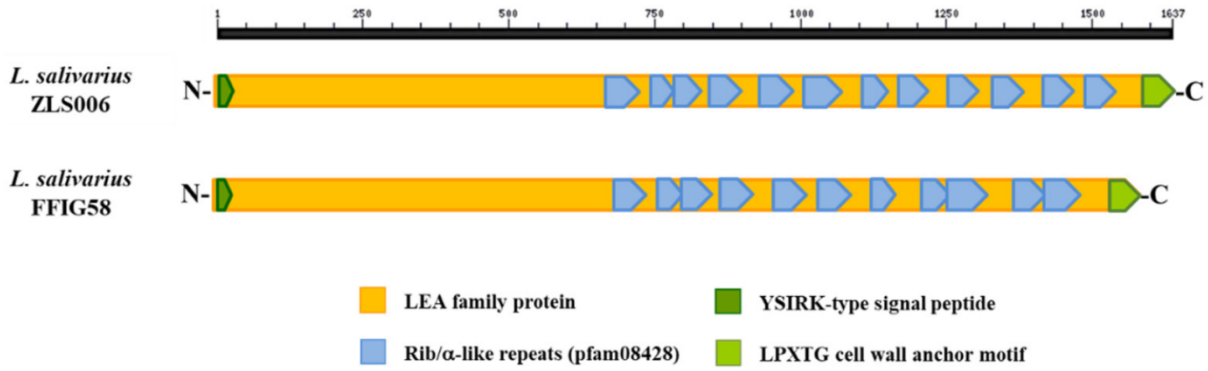


Figure 3-11. Comparison of the domain organization in the LEA family adhesin from *Ligilactobacillus salivarius* FFIG58 isolated from the intestinal mucosa of wakame-fed pigs and the porcine strain *L. salivarius* ZLS006.

3.4. Discussion

In Chapter 2, we discussed that the cell wall and their associated molecules seems to be different in the FFIG strain leading to their different immunomodulatory potentials. The results of this chapter indicate that in addition, these bacterial surface factors would be also related to the different adhesion ability of lactobacilli. Earlier studies demonstrated the importance of lipoteichoic acid as a mediator of the adhesion of lactobacilli to human intestinal epithelial cells[54]. Experiments using purified lipoteichoic acid from the probiotic strain *L. johnsonii* La1 showed that preincubation of the Caco-2 cells with increasing amounts of the pure molecule significantly reduced the adhesion of the La1 strain[54]. On the other hand, it was shown that the EPS-producing strain *L. paracasei* BGSJ2-83 is able to adhere to Caco-2 cells, while the non-producing EPS mutant obtained by insertion mutagenesis of the gene encoding a primary glycosyltransferase has a significantly diminished ability to adhere to intestinal cells[79]. In addition, the BGSJ2-83 strain showed higher adhesion ability than the EPS mutant to HT29-MTX cells. Studying the role of these two bacterial molecules in the adhesion capacity of FFIG strains to PIE cells and mucins, through the generation of mutants, for example, is an interesting topic for future research.

L. salivarius FFIG23 and the FFIG58 sheared a gene for a membrane prepilin peptidase (*comC*) that belongs to the fimbrilin-protein exporter (FPE)

system. The FPE pathway is involved in the assembling of the competence pseudo-pili that is part of the cell-surface appendages in gram-positive bacteria[80]. The competence pseudo-pili is involved in DNA recognition at the cell surface and allows the uptake of exogenous DNA across the bacterial cytoplasmic membrane[81]. The FPE system has been described in several species of lactobacilli[81], but it has not been associated with their adhesion ability to host cells or mucins. As mentioned above, the *L. salivarius* FFIG79 genome contained a putative agglutinin receptor (*ssp5*) or cell surface agglutinin protein that was not found in the genomes of FFIG23, FFIG58, or FFIG63. We further searched for this gene in the other FFIG strains, and we found it only in the genome of *L. salivarius* FFIG124. Some regions of cell surface agglutinin protein in the FFIG79 and FFIG124 strains show similarities with the ssp5 agglutinin receptor of *Streptococcus gordonii*, the isopeptide-forming adherence proteins from *S. oralis*, the antigen I/II family LPXTG-anchored adhesin of *S. orisasini*, and the salivary agglutinin adherence domain-containing protein from *S. mutants*. However, we only found a maximum of 30% similarity of the putative *ssp5* protein in the FFIG strains compared with those found in streptococci. The cell surface agglutinin protein of FFIG79 and FFIG124 strains showed similarities to the antigen I/II family LPXTG-anchored adhesin of *S. orisasini* SH06 in the adhesin P1, the glucan-binding protein C, and the antigen C domains (Fig. 3-12). Blast analysis also revealed a 98.8% of identity (with 68% of query cover) of the

cell surface agglutinin protein of FFIG strains with the putative cell surface agglutinin protein from *L. salivarius* cp400, which also contained an antigen C domain (Fig. 3-12). Of note, FFIG79 and FFIG124 strains significantly differed in their abilities to adhere to mucins and PIE cells. While *L. salivarius* FFIG79 was able to adhere to porcine mucin, the FFIG124 strain was more efficient for the adhesion to PIE cells (Fig. 3-4, 3-5). Then, it is tempting to speculate that the cell surface agglutinin protein of *L. salivarius* FFIG79 and FFIG124 would not have a key role in the colonization of the intestinal tract. It was reported that the antigen I/II family proteins, including the SSP-5 agglutinin, are capable of binding to sialic acid, fucose, lactose, and N-acetylgalactosamine that are abundant in the mucous glycoproteins present in the human saliva, and therefore, are considered important factors that enable the colonization of bacteria in the oral mucosa[82]. It would be of interest to investigate if strains, such as FFIG79 and FFIG124, are capable of colonizing the porcine oral cavity and if they play a positive role in this mucosa.

Fimbria-associated protein 1 (*Fap1*) is a glycosylated surface adhesin required for fimbria biogenesis and biofilm formation in *S. parasanguinis*. The secretion of mature Fap1 is dependent on the presence of the accessory system SecA2-SecY2[83]. Different Fap1-like proteins have been described in several bacterial species[84]. In fact, Fap1-like gene clusters and related glycosylation and secretion loci are present in the genomes of oral streptococci, such as *S. gordonii*,

S. sanguinis, and *S. crispatus*, as well as in the commensal bacteria *L. johnsonii* and *S. salivarius*[85]. These Fap1-like serine-rich proteins have been shown to belong to an expanding family of adhesins known as serine-rich repeats containing adhesins or srr adhesins[85]. In the comparison of FFIG58, FFIG23, FFIG63, and FFIG79, we found a Fap1-like protein (or a srr adhesin) only in the genome of the FFIG58 strain. However, further analysis also revealed the presence of variants of srr adhesins in the genomes of all the remaining FFIG strains.

Since the comparative analysis of the genomes of *L. salivarius* isolated from the intestinal tract of wakame-fed pigs did not allow us to identify unique factors that explain their different abilities to adhere to mucins or epithelial cells, we further investigated several proteins and systems that were described to be involved in the adhesion of lactobacilli.

When the functional domains in the srr proteins of FFIG strains were analyzed, we were able to identify the KxYKxGKxW signal peptide and the serine-rich repeat adhesion glycoprotein AST domain in the N-terminal end, the LPXTG cell wall-anchoring motif at the C-terminus, as well as highly repeated serines in the middle of the molecule. Those sequences have been identified as conserved in the organization of srr proteins, particularly in the adhesins of oral streptococci[86]. However, our analysis did not reveal any known adhesin-associated binding domain the srr proteins of FFIG strains. Our results are in line

with the previous genomic analysis performed by Lee *et al.*[42], who were not able to find srr proteins in the genome of *L. salivarius* of porcine or chicken origins that satisfice all the necessary requirements to consider those proteins as functional adhesins related to the SecA2-SecY2 system. Further studies evaluating the functional properties of the srr proteins and the SecA2-SecY2 system genes in the FFIG strains in the context of adhesion to mucins and PIE cells would be of value to determine their precise role, or the lack of it, in the adhesion capabilities of the *L. salivarius* strains isolated from the intestine of wakame-fed pigs.

Several mucus-binding proteins (MucBPs) have been identified in lactic acid bacteria and were associated with their ability to colonize the gastrointestinal tract[86]. MucBPs contain variable numbers of mub repeats, each of them is divided into two domains, a mucin-binding domain and an immunoglobulin-binding domain[87] [88]. These mub repeats are capable of mediating the adhesion of lactobacilli to mucin glycans through interactions with terminal sialic acid[89] [90] . Up-to seven different MucBP orthologous were found in the pangenome of *L. salivarius*[42]. According to the comparative genomic analysis of FFIG strains and other *L. salivarius* strains isolated from humans, pigs and chickens, we were not able to identify any association between the adhesion ability to mucin or PIE cells and the mucus-biding proteins present in the genomes.

The LEA proteins were detected in all the *L. salivarius* strains isolated from the intestine of wakame-fed pigs and they were also similar to the LEA

proteins found in the genome of *L. salivarius* strains of porcine origin. Proteins with Rib/α-like repeats in lactobacilli have been associated with the binding to the stratified squamous epithelial cells, and consequently, they are considered adhesins facilitating the binding to vaginal epithelial cells[62 91]. A recent study evaluating the temporal variations of gut- associated microbiota in piglets in the first month after birth demonstrated that the small and large intestines sheared a similar composition profile at birth, and while the small intestinal microbiota remained relatively stable, the large intestine quickly expanded and diversified by day 35[92]. Interestingly, the work revealed that the microorganisms from the maternal milk were the main colonizers of the small intestine (approximately 90%), and although the bacteria of maternal milk contributed to 90% of the microbiota in the large intestine after birth, their presence gradually diminished, and they were replaced by fecal microbes derived from mothers by day 35. Then, vertically transmitted maternal milk and intestinal microorganisms would be of key importance in the strengthening of intestinal barrier functions and the development of the mucosal immune system in porcine neonates. Of note, the remaining 10% of the microbial population that initially colonizes the gut of piglets comes from the vagina and the areolar skin of mothers, which also contain diverse bacterial communities. Although the vaginal seeding is transient after the piglet's birth, it was suggested that this initial microbial acquisition from the mother is involved in the preparation of the porcine newborns for host-microbial

symbiosis[93] as it was also suggested for mouse[93] and human[94] neonates. Then, considering that lactobacilli species have been isolated not only from the gastrointestinal tract of pigs, but also from porcine vaginal mucosa and maternal milk[95 96], the presence of the LEA family adhesins in the genome of FFIG strains raise the question of whether these bacteria can also be found in other ecological niches like the vaginal mucosa. The presence of these immunomodulatory bacteria in the various maternal niches could be a mechanism to ensure the colonization of the newborn's gastrointestinal tract by microorganisms that help the immune system to mature. In support of this hypothesis, it was shown that porcine maternally transmitted bacteria had a strong correlation with the expression of antimicrobial peptides, pattern recognition receptors (PRRs), and immunoregulatory cytokines in the guts, highlighting the involvement of maternally derived microorganisms in the maturation of the intestinal immune system.

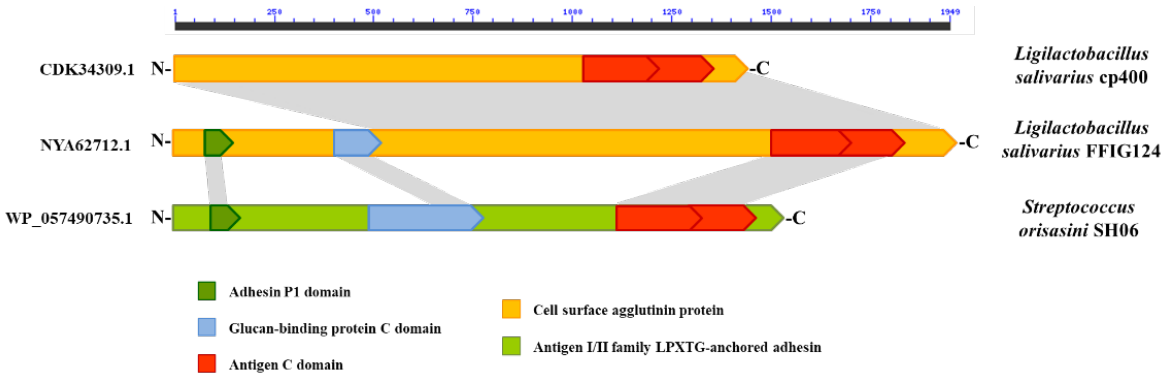


Figure 3-12. Comparison of the domain organization in the putative cell surface agglutinin proteins from *Ligilactobacillus salivarius* FFIG124 and cp400 and the antigen I/II family LPXTG-anchored adhesin from *Streptococcus orisanini* SH06. The antigen I/II family LPXTG-anchored adhesin is a protein of 1,518 amino acids while the putative cell surface agglutinin proteins from the FFIG124 and cp400 strains are proteins of 1,949 and 1,420 amino acids, respectively. Conserved amino acids sequences are shown in gray.

3.5. Summary

In this chapter, human colonic tissues and porcine intestine tissues were first purified to obtain purified soluble human colonic mucin and soluble porcine intestine mucin. Adhesion to mucins and porcine epithelial cells were conducted by the Biacore assay and fluorescent bacteria count, respectively *L. salivarius* FFIG79 showed the higher ability to adhere to soluble mucin while FFIG58 showed the strongest adhesion capacity to PIE cells. There were no significant differences in bacterial surfaces when the SEM analysis was applied to FFIG58 and FFIG79 strains.

The analysis of the genes related to the SecA2-SecY2 secretion system, mucus-binding proteins, pillus operon, and *Lactobacillus* epithelium adhesin (Fig. 3-13) in the lactobacilli isolated from wakame-fed porcine intestine, indicated that there is no specific factor that could explain the different “adhesion phenotypes” of these eight selected strains.

The result of Chapter 2 indicated that differential immunomodulatory activities of FFIG strains in the context of innate immune responses triggered by the activation of TLR3 or TLR4 in PIE cells would be dependent mainly on bacterial surface structures, such as peptidoglycan and EPS. In Chapter 3, our results also showed a different adhesion capacity to porcine mucins and PIE cells for each of the FFIG strains. We were not able to identify any particular molecule

or group of molecules that could explain the different adhesion capabilities of the *L. salivarius* strains isolated from the intestine of wakame-fed pigs. It is tempting to assume that in the same way that occurs with the immunomodulatory activity, the different adhesive capacity of the FFIG strains depends on the combination of several factors acting simultaneously and that have been described to be involved in the adhesion of lactobacilli to the intestinal tract, such as EPS, lipoteichoic acid, MucBP, and other adhesins.

Interestingly, the three *L. salivarius* strains (FFIG23, FFIG53, and FFIG58) capable of efficiently increasing the expression of IFN-β and Mx1 in PIE cells showed different capacities to bind to mucins and PIE cells (Fig. 3-13). In addition, the two strains with the highest capacity to bind to PIE cells (FFIG58 and FFIG63) did not modulate in the same way the response of epithelial cells to the challenge with the TLR3 ligand. While *L. salivarius* FFIG58 showed a remarkable ability to increase IFN-β and Mx1 expression levels, the FFIG63 strain did not modify Mx1 levels and even decreased IFN-β values (Fig. 3-11). Another example is given by the group comprised of the FFIG79, FFIG23, and FFIG53 strains, which all presented a low adhesion to PIE cells. However, while FFIG53 and FFIG23 strains increased the expression of IFN-β and Mx1, *L. salivarius* FFIG79 decreased the values of both factors (Fig. 3-13). Then, the results indicate that there is no correlation between the immunomodulatory capacity and the adhesion ability to mucin and epithelial cells. Therefore, in the

selection of strains destined to colonize the intestinal mucosa and modulate the immunity of the host, both properties must be adequately evaluated. Of note, *L. salivarius* FFIG58 functionally modulated the innate immune responses triggered by TLR3 and TLR4 activation in PIE cells and efficiently adhered to these cells. Therefore, *L. salivarius* FFIG58 is a good candidate as immunobiotic for further *in vivo* studying the protective effect of lactobacilli against intestinal infections in the porcine host.

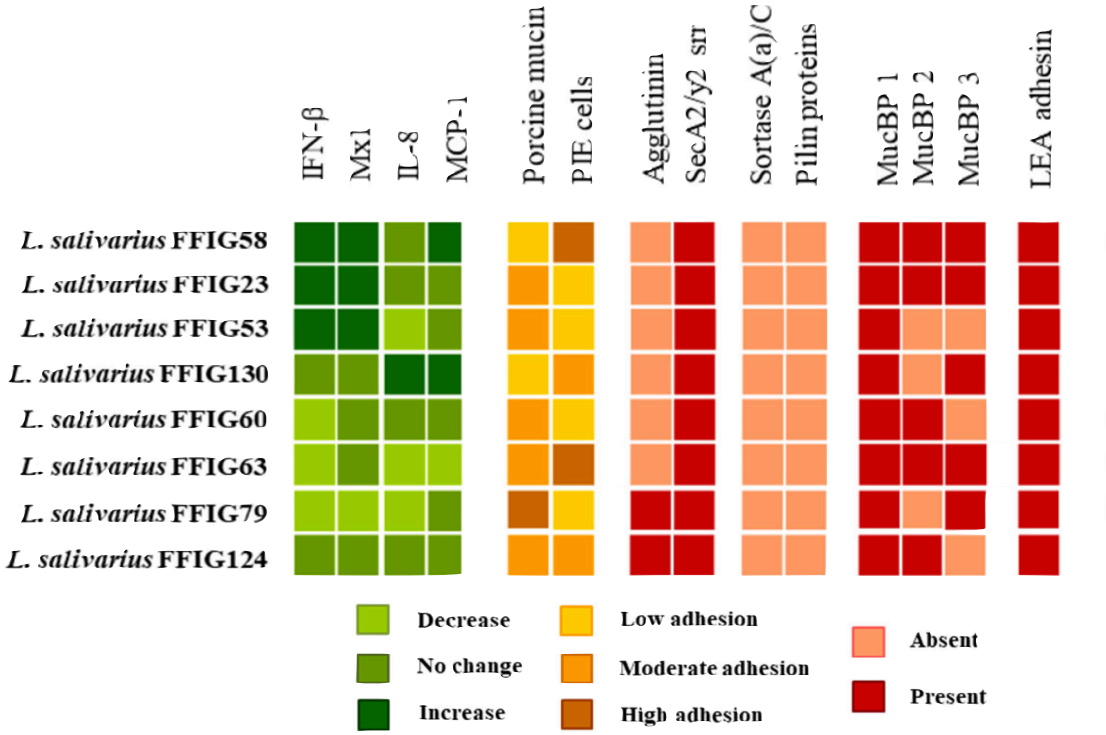


Figure 3-13. Comparison of the immunomodulatory and adhesion capabilities of *Ligilactobacillus salivarius* isolated from the intestinal mucosa of wakame-fed pigs.

Chapter 4

Wakame assimilation ability of isolated lactobacilli and genomic studies

4.1 Introduction

The combination of immunobiotics and immunoprebiotics were thought to be promising candidate as substitutes for antibiotics in livestock. In Chapters 2 and 3, we investigated and discussed the immunomodulatory and adhesion properties of *L. salivarius* isolated from the intestinal tract of wakame-fed pigs. In this chapter, we evaluate and discuss the ability of *L. salivarius* isolates to assimilate the saccharides derived by wakame, in order to develop immunosymbiotics for the porcine host.

Wakame (*Undaria pinnatifida*) belonging to the brown algae, has been widely consumed as edible seaweed in Japan, Korea and other Asian countries. In this regard, the Miyagi Prefecture has become an important producer of wakame in Japan[18]. Wakame contains some bioactive components including fucoidan, alginic acid, cellulose, hemicellulose, omega 3 fatty acid, phlorotannins, vitamins and minerals, which has been reported to have beneficial effects in human host. Among these beneficial effcts it was described that wakame possesses antibacterial, antioxidant and anti-inflammatory functions[97]. Along with the increased consumption of wakame, its side waste products have dramatically been enhanced, becoming a real problem with ecological implications.

Recently, in order to improve growth performance and health of livestock, seaweeds such as wakame, due to their bioactive components, have been studied as feed supplements for animal production, especially in pigs [98]. The addition of

seaweeds to porcine feeds was shown to increase specific IgG in the serum of animals[99]. In particular, Shimazu *et al.* reported that the addition of wakame to porcine feed was capable of increasing the percentage of peripheral blood NK cells and $CD3^{+}CD4^{-}CD8^{+}$ lymphocytes. Additionally, wakame administration influences the intestinal microbial populations by means of increases in the *Lactobacillus* populations and decreases in *E. coli*[18]. Thus, wakame has been considered as promising immunoprebiotics.

In this chapter, in order to develop a most promising immunosynbiotics, the ability of immunobiotic lactobacilli to survive the severe acidic environment in the gastrointestinal tract was evaluated. In addition, the capacity of the lactobacilli strains to assimilate wakame was assessed. Thus, wakame assimilation research was conducted by evaluating turbidity of the fermentation in enzyme-treated wakame, the viable bacterial counts, and the saccharide contents in enzyme-treated wakame. Furthermore, the tolerance to simulated gastric juice were studied for the selected lactobacilli strains.

4.2 Materials and Methods

4.2.1 Preparation of wakame broth

Fine wakame powder was suspended in distilled water to make a 0.1% suspension. This suspension was autoclaved at 121 °C for 15 min and subjected for initial enzymatic hydrolysis. For hydrolysis, wakame broth was first adjusted to pH 5, which was determined as the optimal pH for enzymes activities. Then, wakame was incubated at 50 °C for 24 h with the two enzymes: cellulase (0.5%), hemicellulase (0.5%) (Mitsubishi-chemical foods corporation, Tokyo, Japan). The hydrolyzed wakame broth was again autoclaved at 121 °C for 15 min, and the supernatant was separated by centrifugation at 6000 rpm for 20 min. The supernatant containing the wakame extract was further supplemented with yeast extract (0.1%) and NaCl (0.5%). The pH was adjusted to 6.8 and the broth was autoclaved at 121 °C for 15 min. The wakame broth obtained at the end of these procedures was considered as ready for use. In order to prepare the wakame agar medium, 1.5% agar was added to the wakame broth.

4.2.2 Bacteria culture condition and OD value

The lactobacilli strains used for in wakame assimilation studies were first grown in MRS agar at 37 °C for 24 h. A single colony was picked for sub-culture. After growth, 2% (v/v) of this culture was used for sub-culture into the newly developed wakame-broth and incubated at 37 °C for 24 h. Starter culture was

inoculated into 5 mL of three same wakame broth using L-shaped test tubes with optical density (OD) = 0.5 inoculated over time (measurement interval: 30 min, penetration rate: 60 rpm, total operation time: 24 h) (TVS026CA, Advantec, Tokyo, Japan). Bacterial viability was performed by plate counting on MRS agar plates. Strains used in this chapter were showed in Table 4-1

4.2.3 Prolonged fermentation

Wakame sub-cultured bacteria were inoculated in new wakame broth. The pH was adjusted to 6.8 after 12, 24, 48, and 72 h, by using 0.1N NaOH. The pH and the OD values were evaluated each time before pH adjustment. In addition, the bacterial viability was assessed by colony counting on MRS agar plate in each time point. TLC analysis was also performed as described below. Samples for the evaluation of saccharides assimilation were taken at 24, 48, 72, 96, 120 and 144 h.

4.2.4 Separation and hydrolysis of saccharides in enzyme-treated wakame solution

Separation of saccharides in enzyme-treated wakame solution was conducted by gel filtration chromatography with a Bio-Gel P2 extra fine column (90 × 2.6 cm; BIO-RAD, California, USA) using degassed water (ultra-sonic treatment) as the mobile phase. The presence of saccharides were confirmed by TLC analysis as described below. The fractions containing the different saccharides were collected and lyophilized for further study.

Disaccharides (100 μL) were hydrolyzed by adding 100 μL 8M of trifluoroacetic acid for 3 h reaction at 100 °C. The hydrolyzed disaccharides were analyzed by TLC analysis.

4.2.5 TLC analysis

Thin layer chromatography (TLC) analysis was performed as follow. Wakame solution with or without enzyme treatment, the supernatant of the wakame broth fermented with lactobacilli, and 1% (w/v) of standard saccharides solutions (glucose, galactose, xylose, fructose, sucrose, fucose, cellobiose, lactose, raffinose, and galactooligosaccharide) were dropped on TLC Silica gel 60 (Merck kGaA, Darmstadt, Germany). Butyl alcohol, isopropyl alcohol and milli Q (3:12:4) mixture was used as solvent. After 2 times developing, 5% (v/v) sulfuric acid in methanol was sprayed on the plate and it was heated at 150 °C for 10 min until spot visualizing. R_f value was calculated as the following formula:

$$R_f = \frac{\text{Distance traveled by sample}}{\text{Distance traveled by solvent}}$$

The mean values of spots were analyzed by ImageJ (https://imagej.nih.gov/ij/).

4.2.6 Survival of lactobacilli in simulated gastric juice

Bacterial survival was evaluated in the simulated gastric juice, which contained 25 mg/mL of pepsin (2800 units/mg, from porcine stomach, Fujifilm Wako Pure Chemical Corporation). The pH was adjusted to 2, with 0.1N HCl.

Wakame solution with enzyme treatment was added to gastric juice (1%(v/v)) for the experiment and sterilized MilliQ water was added as the control. Three times sub-cultured bacteria were incubated in MRS broth for 16 h. Fermented solution was centrifuged at 6000 rpm for 10 min and the supernatant was removed twice. The simulated gastric juice with or without wakame solution was added to the pellet and bacteria were suspended by Vortex. Bacterial viability was performed by plate counting on MRS agar plates after 0.5, 1, 2, 3, 4, and 5 hours.

4.2.7 Scanning electron microscope

Cultured bacteria was washed once and diluted 2 or 3 times with PBS. The bacterial suspension was dropped on a polycarbonate membrane (0.2 μm, ADVANTEC) and filtered with vacuum filtration (Millipore). The membrane with bacteria on its surface was immersed in 2% (v/v) glutaraldehyde solution. After 1 h, the membrane was immersed in 50, 60, 70, 80, 90 and 99% ethanol in turns of 20 min in order to remove water. The membrane was finally immersed in t-butyl alcohol and lyophilliized. Platinum palladium was deposed and the sample was submitted for SEM (HITACHI) observation.

Figure 4-1. Wakame powder. Left: leaf wakame powder. Right: stalk wakame powder.

Table 4-1. Species, strains and origins of the bacteria used in this chapter.

Species	**Strain**	**Origin (porcine)**
Ligilactobacillus salivarius	FFIG23	Ileum peyer's patch
Ligilactobacillus salivarius	FFIG53	Jejunum
Ligilactobacillus salivarius	FFIG58	Jejunum
Ligilactobacillus salivarius	FFIG60	Jejunum
Ligilactobacillus salivarius	FFIG63	Jejunum
Ligilactobacillus salivarius	FFIG79	Ileum peyer's patch
Ligilactobacillus salivarius	FFIG124	Ileum
Ligilactobacillus salivarius	FFIG130	Ileum
Lactiplantibacillus plantarum	VG 137	Vagina
Lactiplantibacillus plantarum	BC 74	Vagina
Lactiplantibacillus plantarum	4FeB 132	Fece of piglet
Lactiplantibacillus plantarum	$4M_4326$	Milk (4 week)
Lactiplantibacillus plantarum	$4M_4338$	Milk (4 week)

4.3 Results

4.3.1 Wakame assimilation ability of isolated lactobacilli

Selected *L. salivarius* strains were allowed to grow in two types of wakame broths developed by our group: a) containing the wakame leaf or, b) containing the wakame stalk. In both mediums, wakame was enzyme-treated. The turbidity of fermentation in leaf wakame broth ranged from 0.4 to 0.5, while in stalk wakame broth reached values from 0.45 to 0.6 (Fig. 4-2). *L. salivarius* FFIG79 obtained the highest OD value (0.512), while FFIG60 had lowest OD value (0.401) when the fermentation in leaf wakame was evaluated. When the fermentation in stalk wakame was analyzed, it was observed that FFIG124 obtained the highest OD value (0.599), while FFIG58 showed lowest OD (0.463). The comparison of the growth rate and lag time during 24 h fermentation, showed that *L. salivarius* FFIG79 grew faster in leaf wakame broth while FFIG60 showed the slowest growth rate (Table 4-2). However, there was no significant differences between growth rate of 8 strains when grew in stalk wakame broth.

Prolonged fermentation experiments are designed by pH modulation in order to investigate saccharide consumption. Samples were taken at 12, 24, 48 and 72 h for the determination of pH and OD values as well as viable bacterial counts. The pH was kept at a low level after 72 h when the 8 strains of *L. salivarius* were used to ferment both wakame broth (Table 4-3). In leaf wakame, *L. salivarius* FFIG124 had higher OD value from 12 h to 72 h while FFIG79 had lower OD

value at 48 h. FFIG23, FFIG58 and FFIG63 had the most viable bacteria (Fig. 4-3AB). In stalk wakame, *L. salivarius* FFIG63 had higher OD value at 72 h while FFIG23 had lower OD value at 72 h. *L. salivarius* FFIG60, FFIG79 and FFIG130 had the most viable bacteria (Fig. 4-3CD).

Samples for the evaluation of saccharides assimilation were taken at 24, 48, 72, 96, 120 and 144 h. Glucose, galactose, cellobiose, lactose, raffinose and galactooligosaccharide were used as standard saccharides. Both, monosaccharides and disaccharides were detected in both leaf and stalk enzyme-treated wakame broths (Fig. 4-4AB). It was observed that after 72h, the color of spots were faded and close to white, indicating that both types of saccharides were decreased in the two wakame mediums (Fig. 4-4AB). Then, the mean value of each spot was measured by Image J analysis. In leaf wakame, FFIG58, FFIG60, FFIG63 and FFIG79 strains were able to utilize both monosaccharides and disaccharides better than the other strains. The mean value of monosaccharide fermentation for the FFIG53 strain did not decreased while the value of the disaccharide had a significant decrease. The FFIG130 strain was also able to assimilate disaccharides better than monosaccharides (Fig. 4-5). Compared to leaf wakame, after 72 h, the mean values of spots of all the lactobacilli strains were significantly decreased (Fig. 4-6). The mean value of both saccharides after the fermentation with FFIG63 and FFIG124 strains was closed to 1 after 72 h, which meant that the spot color was very close to white color. These results indicated

that both saccharides were almost completely consumed. In addition, it was observed that the FFIG60 and FFIG130 strains could utilize disaccharides better than monosaccharides in the stalk wakame broth (Fig. 4-6). These results indicated that the ability of the eight FFIG strains to utilize carbon sources (monosaccharides and disaccharides) is better in the stalk wakame broth than in the leaf wakame medium. The reason for these results could be related to the fact that these lactobacilli strains were isolated from pig fed with stalk wakame.

4.3.2 Saccharide in enzyme-treated wakame solution

As described previously, both monosaccharides and disaccharides were detected in the enzyme-treated wakame solutions. In order to determine the types of saccharides present in the enzyme-treated wakame broth, we separated the saccharides by gel filtration chromatography. We collected 110 fractions for both wakame solutions. Two components with different molecule weights were separated (Fig.4-7). Fractions 30 ~ 35, and 65 ~ 72 of leaf wakame, and fractions 28 ~ 35 and 65 ~ 71 of stalk wakame were collected and lyophilized.

In order to identify the disaccharide, we hydrolyzed the disaccharide by adding trifluoroacetic acid (Fig. 4-8). According to the R_f value, the disaccharide in wakame had a high possibility to be cellobiose and the hydrolyzed disaccharide in both wakame solutions showed similar polarity characteristics with glucose and fructose (Table 4-4).

4.3.3 Morphology of isolated *L. salivarius* strains and other wakame assimilative lactobacilli

Five *Lactiplantibacillus plantarum* strains isolated from porcine milk, vagina and feces were evaluated in relation to their wakame assimilation abilities. These 5 strains showed higher OD values (0.8 ~ 0.9) than *L. salivarius* strains (Fig. 4-9) when wakame assimilation experiments were performed. Compared to *L. salivarius* strains, *L. plantarum* grew faster and the lag time was shorter when experiments were performed in leaf wakame (Table 4-5). Although *L. salivarius* FFIG58 showed the earliest lag time in MRS broth compared to all the lactobacilli strains, *L. plantarum* had a better wakame assimilation ability. In particular, *L. plantarum* 4m4326 showed the shortest lag time (3.077 h) while the 4M4 338 strain showed the fast growth rate when fermented in leaf wakame broth.

By comparing the SEM photos, *L. plantarum* have longer cell body than *L. salivarius.* When grown in wakame broth, bacteria bent and became longer and swelling surrounded by polysaccharide (Fig. 4-10). Both strains had poor ability of split in wakame broth compared to MRS medium.

4.3.4 Genomic characterization of *L. salivarius* strains with different abilities to grow in wakame

It was reported *L. salivarius* strains have different abundance of genes belonging to glycosyltransferases and glycosylhydrolases families[43]. The work

highlighted that the set of glycosyltransferases and glycosylhydrolases among each strain could confer them variable saccharides metabolic pathways that would be associated to their different abilities to interact with abiotic and biotic environmental factors. In particular, the set of glycosylhydrolases could determine the carbon sources that each strain can use for growing. Then, we also evaluated the abundance of genes belonging to glycosylhydrolases families among the FFIG strains and compared them with other *L. salivarius* strains of animal origin (Fig. 4-11).

Interestingly, the clustering analysis considering the numbers and types of glycosylhydrolases showed that strains FFIG58, FFIG63, FFIG79 and FFIG124 had a significant higher abundance of enzymes from the families GH25 and GH13, when compared with all the other *L. salivarius* strains. Of note, among the strains evaluated in this work, *L. salivarius* FFIG124 and FFIG63 stood out for its ability to grow in stalk wakame-based medium while the FFIG58 and FFIG79 strains showed a better capacity to grow in leaf wakame-based broths than other lactobacilli. Then, these four strains were selected for comparative genomic studies (Fig. 4-12).

The comparative study of the four strains revealed a coregenome of 1,097 genes. The strains with the ability to efficiently grown in wakame stalk, *L. salivarius* FFIG63 and FFIG124 had 54 and 66 unique genes, respectively; while they sheared 16 genes. Among the unique genes of *L. salivarius* FFIG124 we

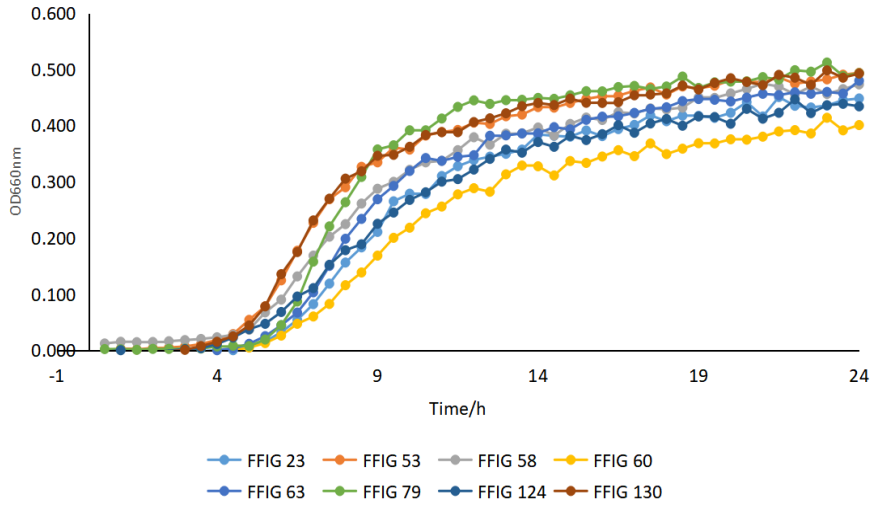
found a UDP-N-acetylglucosamine 2-epimerase, while the two strains sheared a pyruvate kinase. In addition, the strains with the ability to efficiently grown in wakame leaf, *L. salivarius* FFIG58 and FFIG79, had 137 and 48 unique genes, respectively; while they sheared 62 genes. Among the unique genes of *L. salivarius* FFIG58 we found O-acetyl-ADP-ribose deacetylase and a phosphorylated carbohydrates phosphatase while in the unique genes of the FFIG79 strain we found a phosphoglucomutase. The two strains sheared a lactate utilization protein A, a 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase, a phosphoenolpyruvate carboxykinase, a lactose permease, and a putative acyl-CoA thioester hydrolase. These results highlight the different metabolic potential of each strain for using carbon sources.

Of note, when the number of genes involved in carbohydrates metabolism was performed by using the RAST analysis (Fig. 4-13), it was shown that *L. salivarius* FFIG58 had the highest numbers of genes (146) while the FFIG79 had the lowest (136). This would suggest that the differences between strains are not due only to the different genes present in each genome but in addition due to differences in their expression. More studies are necessary to find out the metabolic differences that explain the distinct capacity of the strains to grow in wakame-based mediums.

4.3.5 Tolerance to simulated gastric juice

The ability of surviving in acidic gastric environment is considered as prerequisite when selecting promising probiotic strains. We designed a simulated gastric juice with pepsin and pH adjusted to 2. Viable bacteria were counted at 0, 0.5, 1, 2, 3, 4 and 5 h. We chose the strain FFIG58 that has the best adhesion ability to PIE cells to evaluate how wakame affects the survival of lactobacilli in the gastric juice. Viable cells of the FFIG58 strain were increased when both wakame broths were included in the simulated gastric juice experiments (Fig. 4-14). The SEM analysis allowed finding out that *L. salivarius* FFIG58 without gastric juice stimulation showed smooth surface and plump cell body (Fig. 4-15). However, when the FFIG58 strain was stimulated with the gastric solution for 5 h, the cell body became uneven and the cell surface was destroyed due to the low pH. This alteration induced the material inside the cells to flood out (Fig. 4-15), and caused the death of the bacteria. On the other hand, when the FFIG58 strain was challenged with gastric juice in the presence of stalk wakame, a better preservation of bacterial characteristics was observed. It is tempting to speculate that monosaccharide could maintain an apropiate metabolic activity of the bacteria and that the polysaccharide in wakame could protect it from the low pH environment. Of note, in leaf wakame experiments this protective phenomenom was not observed.

A



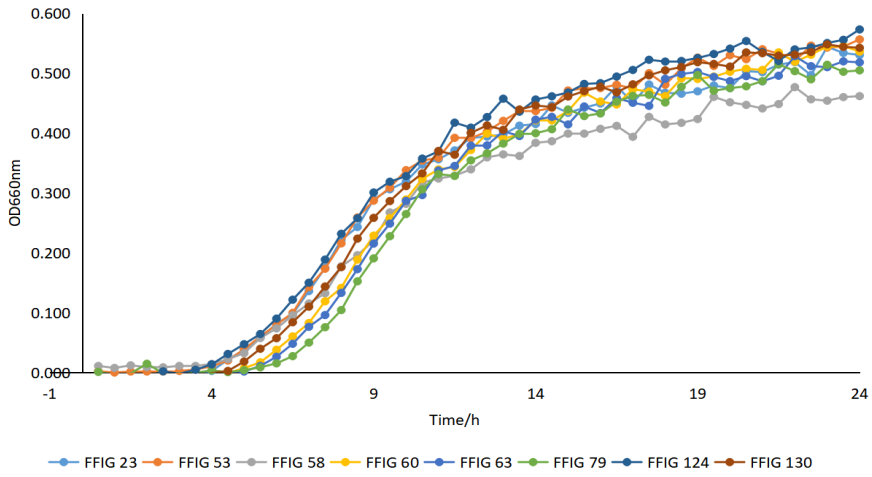


Figure 4-2. Growth curves of *Ligilactobacillus salivarius* isolated from the intestinal mucosa of wakame-fed pigs grown on wakame broth containing enzyme-treated wakame leaf (A) or wakame stalk (B). The results represent data from three independent experiments.

Table 4-2. Growth rate (/h) and lag time (h) of *Ligilactobacillus salivarius* isolated from the intestinal mucosa of wakame-fed pigs fermented in leaf wakame and stalk wakame.

Broth	FFIG23		FFIG53		FFIG58	
	Growth rate (/h)	Lag time (h)	Growth rate (/h)	Lag time (h)	Growth rate (/h)	Lag time (h)
Wakame (leaf)	0.0621	5.501	0.0639	3.772	0.0615	4.881
Wakame (stalk)	0.0618	4.649	0.0637	4.691	0.0531	4.684

Broth	FFIG60		FFIG63		FFIG79	
	Growth rate (/h)	Lag time (h)	Growth rate (/h)	Lag time (h)	Growth rate (/h)	Lag time (h)
Wakame (leaf)	0.0441	5.810	0.0681	5.709	0.0909	5.734
Wakame (stalk)	0.0635	5.496	0.0623	5.645	0.0626	5.935

Broth	FFIG124		FFIG130	
	Growth rate (/h)	Lag time (h)	Growth rate (/h)	Lag time (h)
Wakame (leaf)	0.0485	4.979	0.0722	4.632
Wakame (stalk)	0.0614	4.436	0.063	5.079

Table 4-3. Changes of pH in the cultures in leaf wakame (A) and stalk wakame (B) when fermented with *Ligilactobacillus salivarius* were isolated from the intestinal mucosa of wakame-fed pigs after 0, 12, 24, 48 and 72 h.

A

Time(h)	FFIG23	FFIG53	FFIG58	FFIG60	FFIG63	FFIG79	FFIG124	FFIG130
0	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8
12	4.3	4.2	4.4	4.4	4.3	4.3	4.3	4.2
24	3.7	3.8	3.7	3.8	3.9	3.8	3.8	3.9
48	4.3	4.2	4.2	4.2	4.3	4.5	4	4.3
72	4.5	5.2	4.7	4.7	5.4	4.8	4.5	5

B

Time(h)	FFIG23	FFIG53	FFIG58	FFIG60	FFIG63	FFIG79	FFIG124	FFIG130
0	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8
12	4.6	4.3	4.5	4.5	4.4	4.7	4.1	4.5
24	4.2	4	4.1	4.1	4.1	4.2	3.6	4.1
48	4.1	4	4	3.9	3.8	4.1	3.8	3.8
72	4.6	3.9	4.5	3.8	4.2	4.6	4.3	4.1

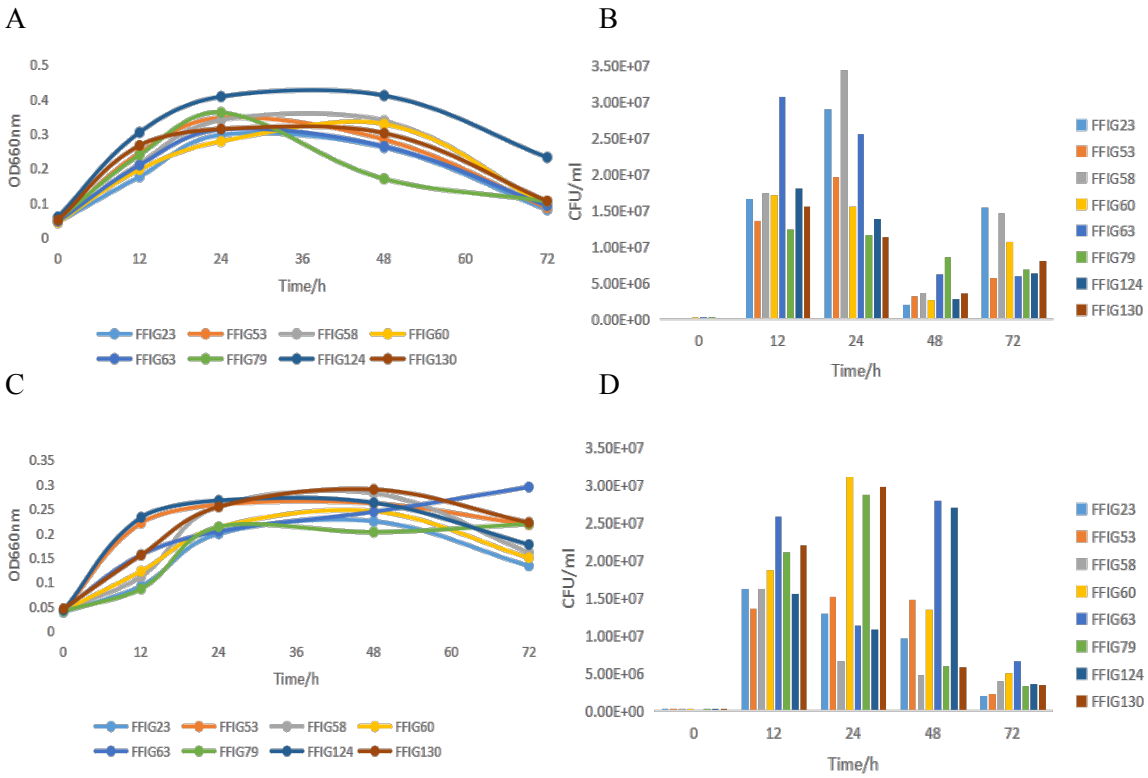


Figure 4-3. Growth curves (AC) and viable bacteria (BD) were evaluated when *Ligilactobacillus salivarius* were isolated from the intestinal mucosa of wakame-fed pigs fermented in leaf wakame (A) and stalk wakame (B) after 0, 12, 24, 48 and 72 h.

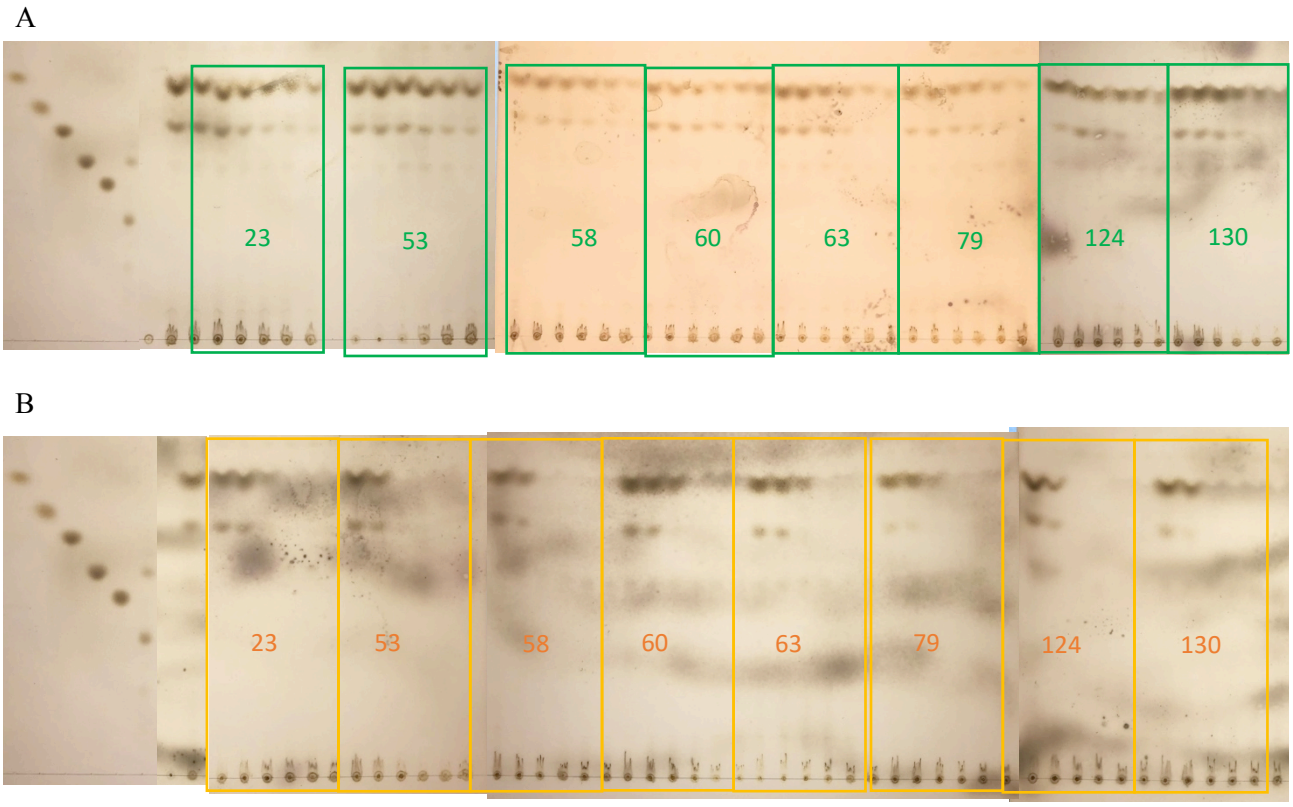


Figure 4-4. Sugar utilization by *Ligilactobacillus salivarius*, isolated from the intestinal mucosa of wakame-fed pigs. Saccharides in the wakame broth cultures of *L. salivarius* strains were analyzed by TLC. From left: standard: glucose, galactose, cellubiose, lactose, raffinose and galactooligosaccharide; wakame before enzyme treatment and wakame after enzyme treatment. Samples were taken after 24, 48, 72, 96 120 and 144 h of 8 strains.

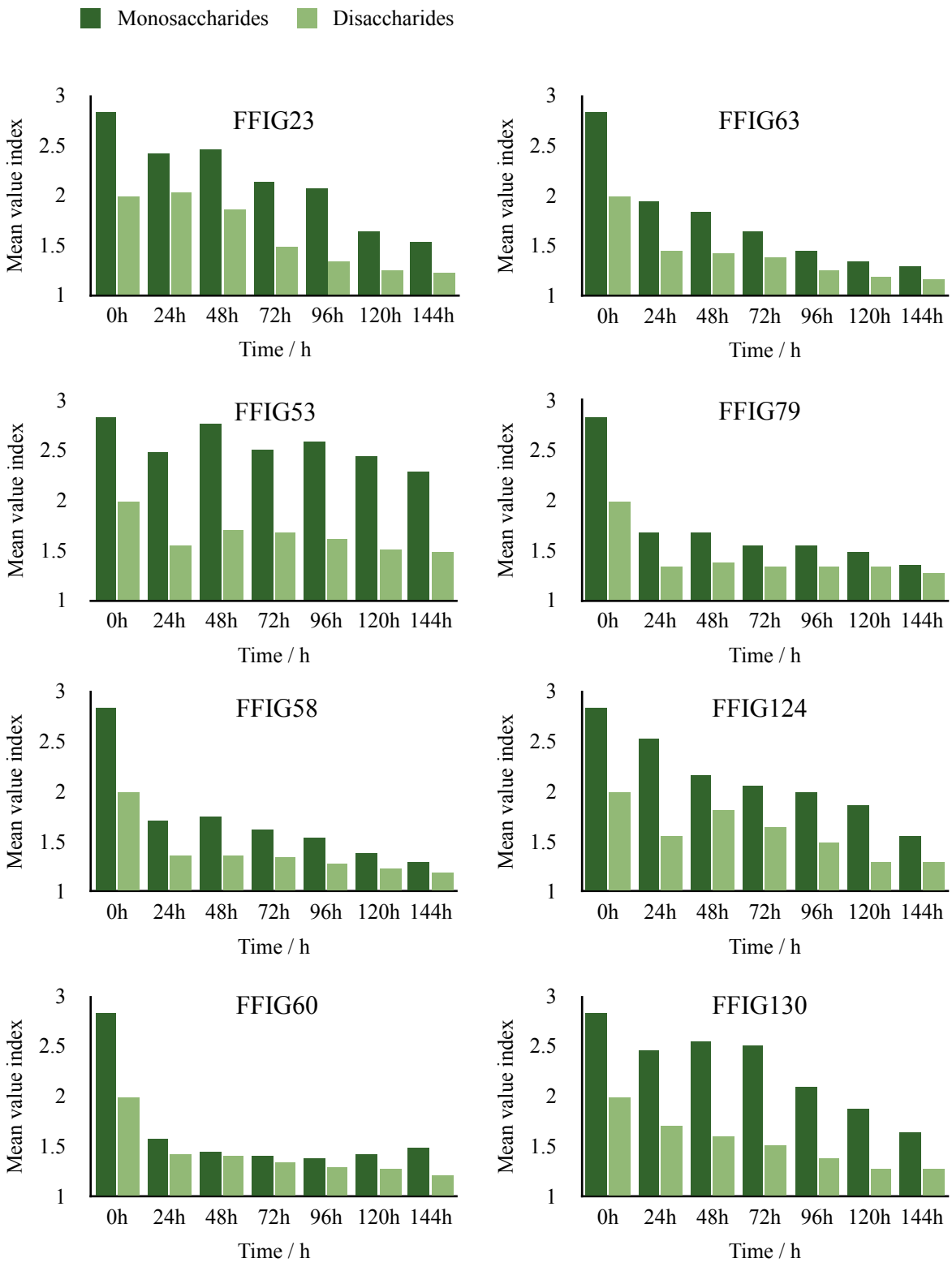


Figure 4-5. Mean value index of spots showed in Fig.4-4 of leaf wakame fermented by *Ligilactobacillus salivarius*. Mean value index = (Mean value of white color)/(Mean value of spot). Mean value was measured by Image J. Lower mean value index (≥ 1) means the spot is closer to white color and higher mean value index (≤ 255) means the spot is closer to black color.

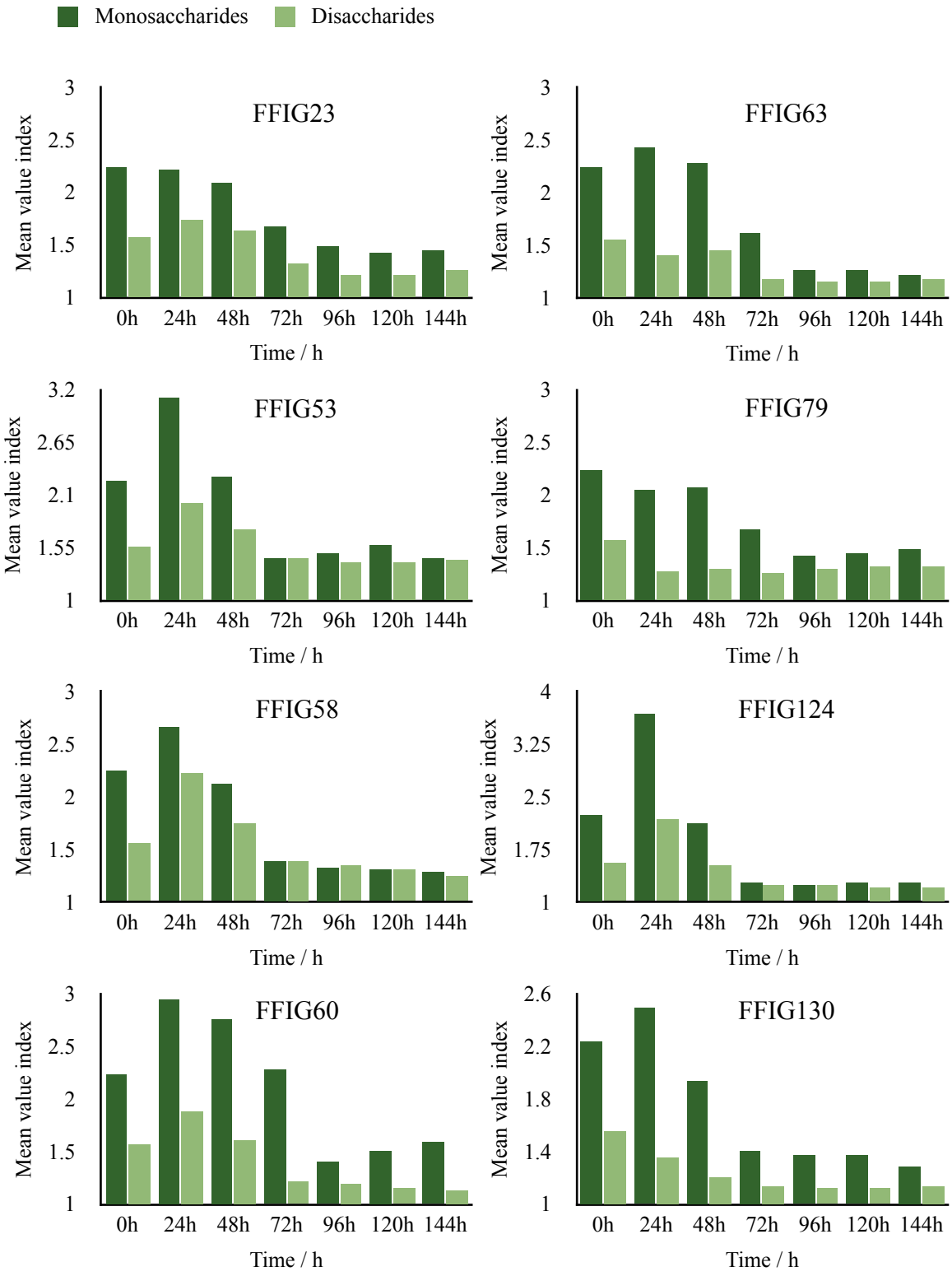
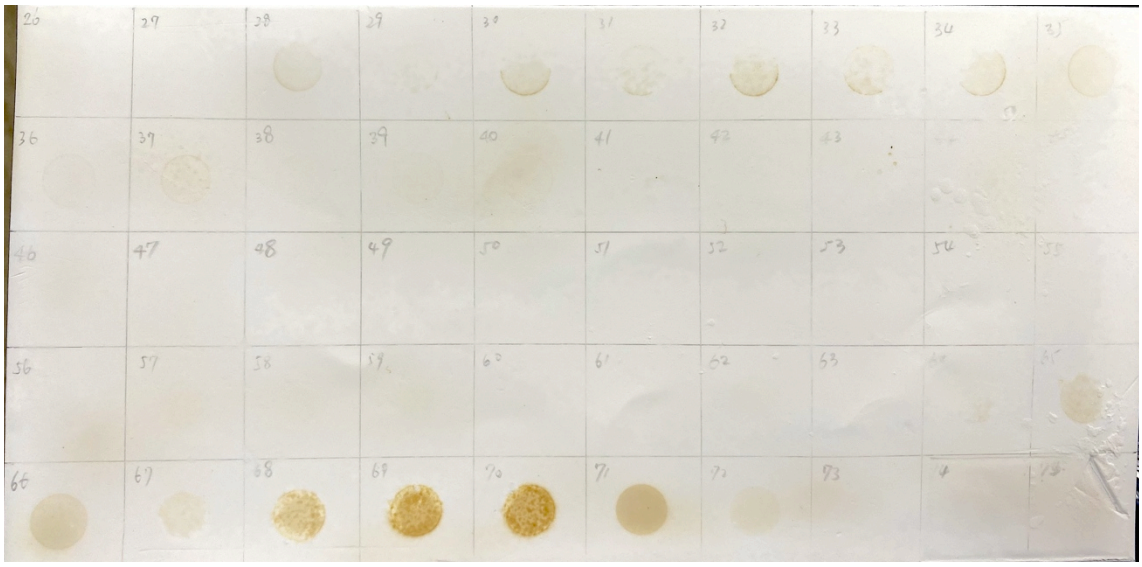


Figure 4-6. Mean value index of spots showed in Fig.4-4 of stalk wakame fermented by eight *Ligilactobacillus salivarius*. Mean value index = (Mean value of white color)/(Mean value of spot). Mean value was measured by Image J. Lower mean value index (≥ 1) means the spot is closer to white color and higher mean value index (≤ 255) means the spot is closer to black color.

A



B

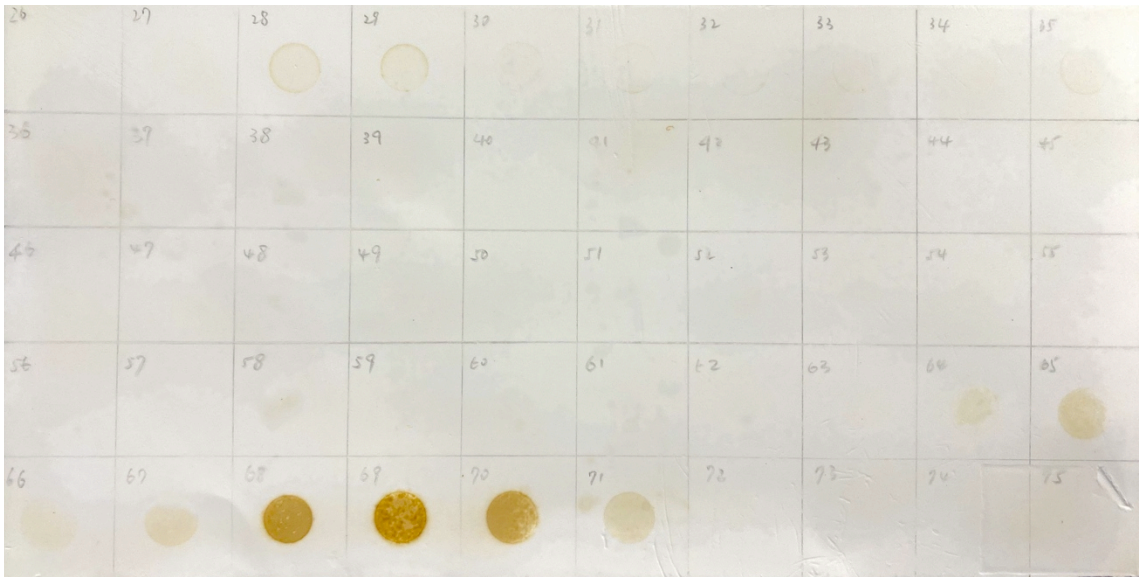


Figure 4-7. TLC analysis of fractions separated by gel filtration chromatography of saccharides in leaf wakame (A) and stalk wakame (B).

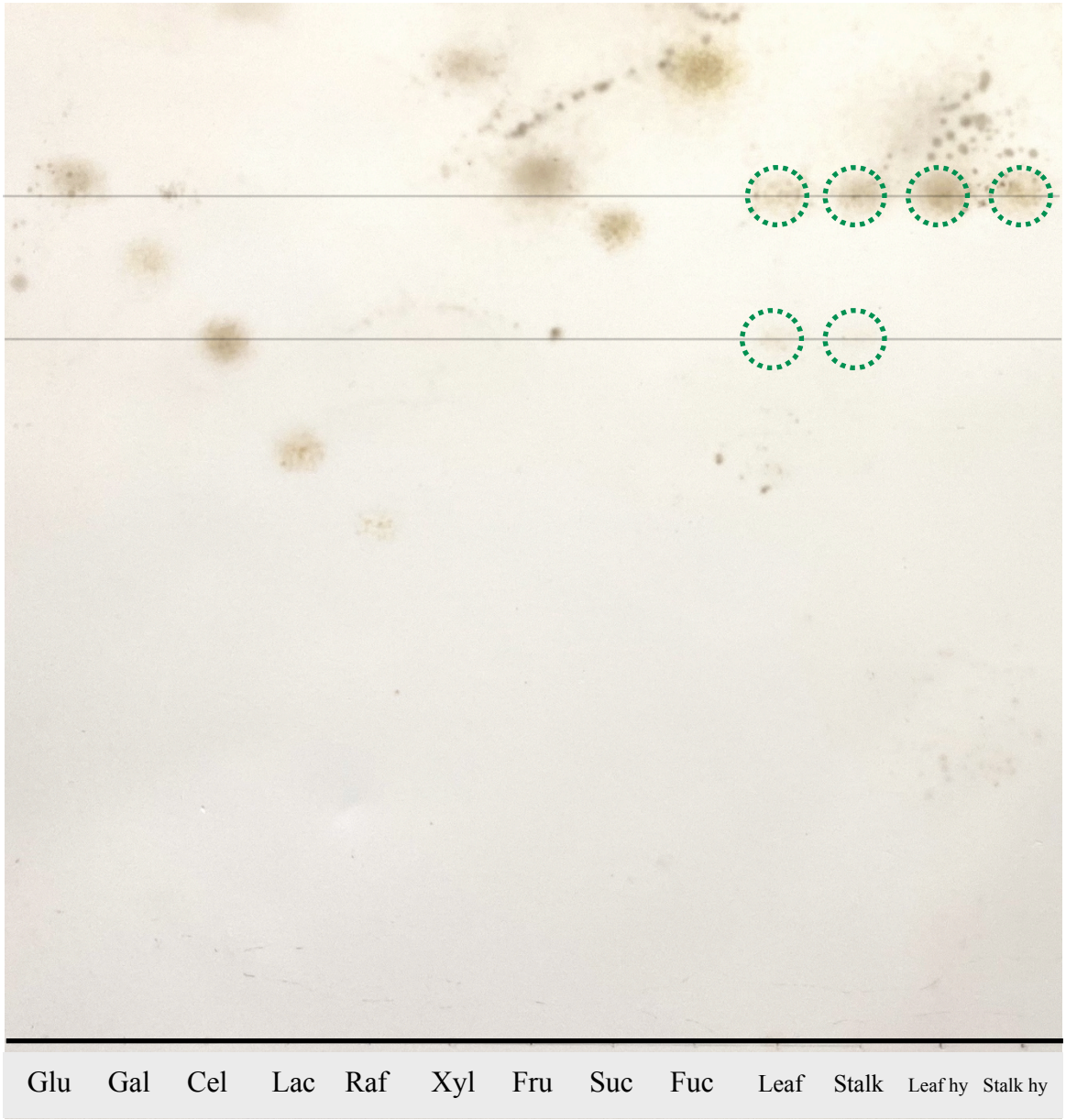


Figure 4-8. TLC analysis of hydrolyzed disaccharide in leaf wakame and stalk wakame. From left: 1% glucose solution, 1% galactose solution, 1% cellobiose solution, 1% lactose solution, 1% raffinose solution, 1% xylose solution, 1% fructose solution, 1% sucrose solution, 1% fucose; leaf wakame after enzyme-treatment, stalk wakame after enzyme-treatment, hydrolyzed disaccharide in leaf wakame, hydrolyzed disaccharide in stalk wakame.

Table 4-4. R_f value according to spots shown in Fig. 4-8

Sample	R_f values	
Glucose	0.668	
Galactose	0.607	
Cellobiose	0.542	
Lactose	0.455	
Raffinose	0.400	
Xylose	0.758	
Fructose	0.669	
Sucrose	0.632	
Fucose	0.752	
Leaf wakame AE	0.659	0.543
Stalk wakame AE	0.659	0.543
Leaf wakame AE hydrolysis	0.662	
Stalk wakame AE hydrolysis	0.662	

AE: After Enzyme-treatment

Table 4-5. Growth rate (/h) and lag time (h) of *Ligilactobacillus salivarius* and *Lactiplantibacillus plantarum* isolated from the porcine intestine, feces, and milk of wakame-fed pigs fermented in leaf wakame and stalk wakame.

Carbon sources	FFIG58	
	Growth rate (/h)	Lag time (h)
MRS	0.2973	2.145
Wakame (leaf)	0.1473	4.713

Carbon sources	VG 137		BC 74		4FeB 132	
	Growth rate (/h)	Lag time (h)	Growth rate (/h)	Lag time (h)	Growth rate (/h)	Lag time (h)
MRS	0.4205	4.963	0.4039	4.582	0.427	4.889
Wakame (leaf)	0.258	3.300	0.2997	3.723	0.2917	4.676

Carbon sources	$4M_4$ 326		$4M_4$ 338	
	Growth rate (/h)	Lag time (h)	Growth rate (/h)	Lag time (h)
MRS	0.4048	4.925	0.4027	5.022
Wakame (leaf)	0.314	3.077	0.322	3.491

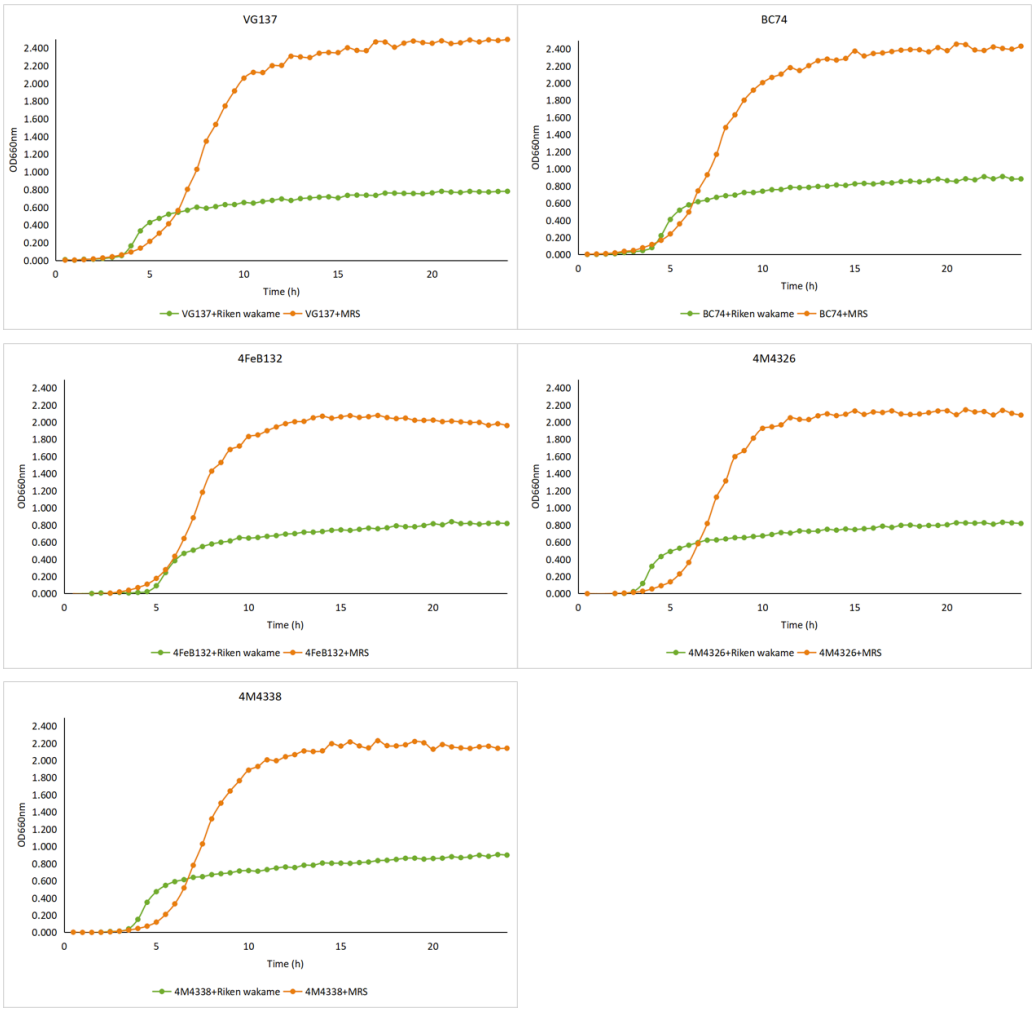


Figure 4-9. Optical density (OD) of *Lactiplantibacillus plantarum* isolated from milk, vagina, feces of pigs grown in leaf wakame. The results represent data from three independent experiments.

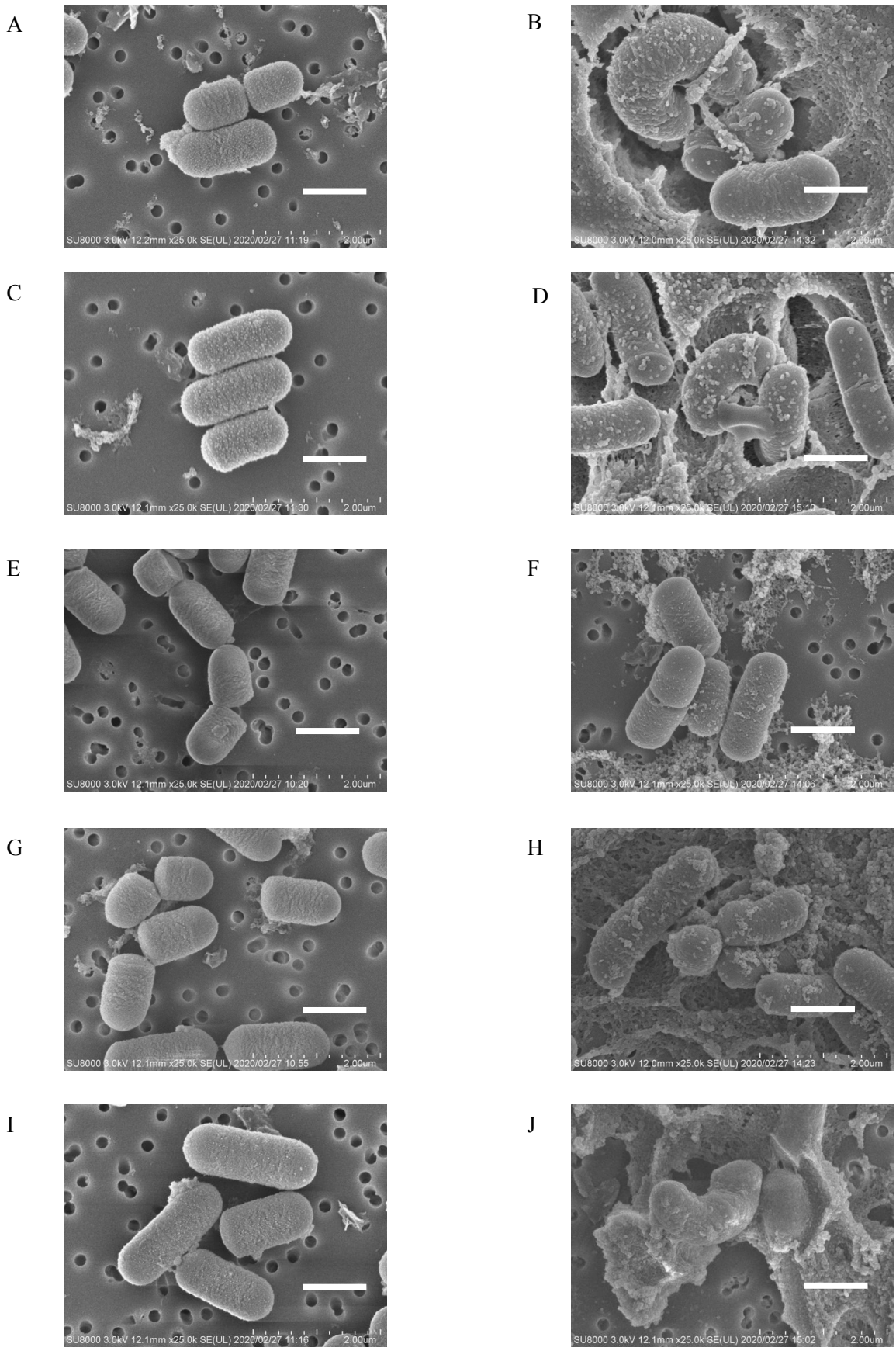


Figure 4-10. Scanning electron micrographs of *Lactiplantibacillus plantarum* 4M$_4$ 326 (A, B), *L. plantarum* 4M$_4$ 338 (C, D), *L. plantarum* VG137 (E, F), *L. plantarum* BC74 (G, H), and *L. plantarum* 4FeB132 (I, J) fermented in MRS (A, C, E, G, I) and leaf wakame broth (B, D, F, H, J) at 37°C after 24 h. Scale bar: 1 μm.

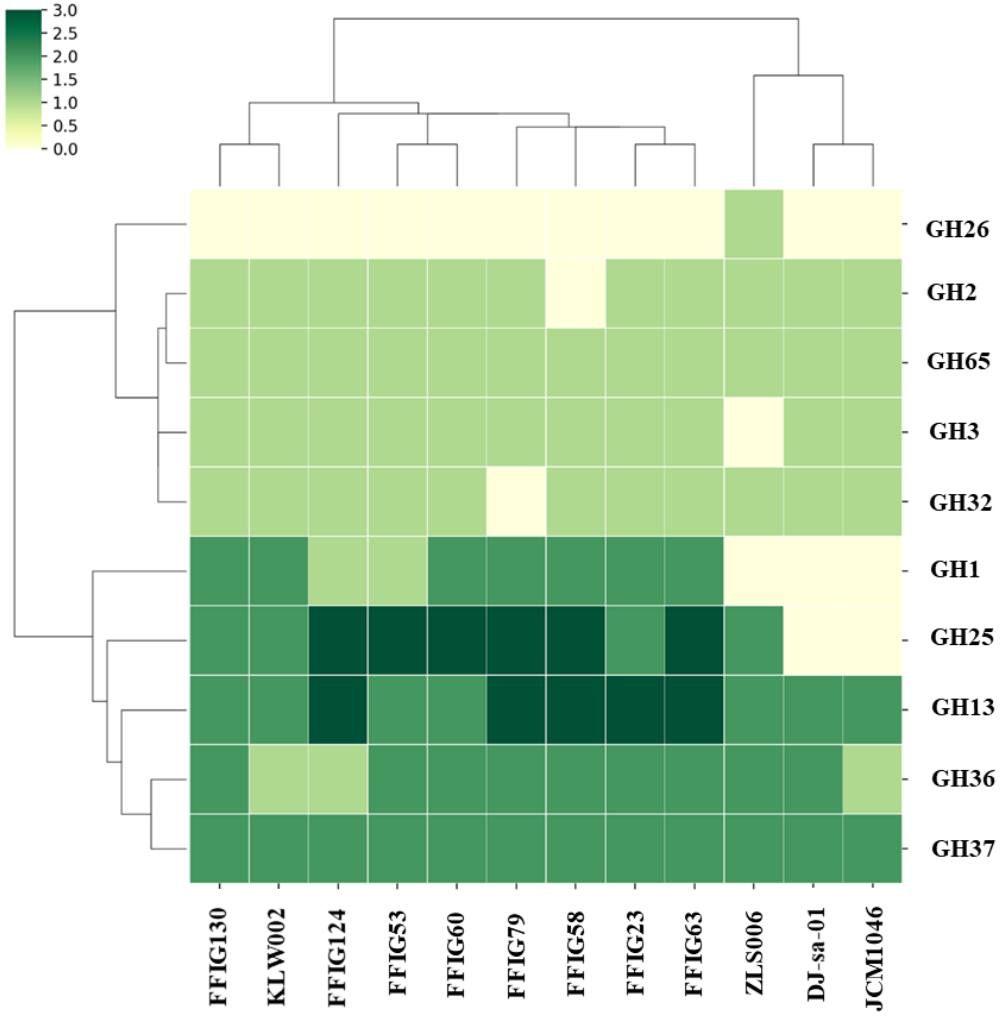


Figure 4-11. Genomic comparison of the glycosylhydrolases present in the genomes of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs and other *L. salivarius* strains of porcine or chicken origin with available public genomes. The heat-map was constructed considering the numbers of glycosylhydrolases in each family.

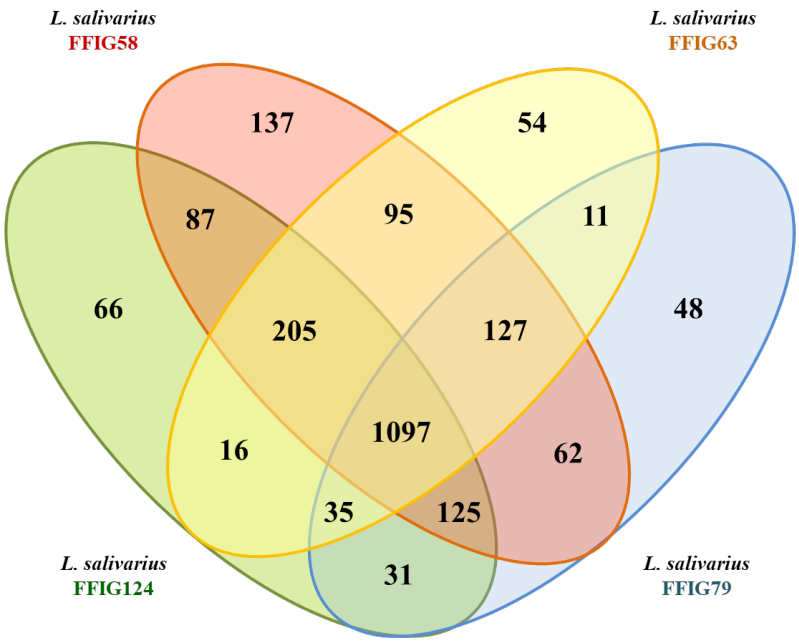


Figure 4-12. Genomic comparison of *Ligilactobacillus salivarius* strains isolated from the intestinal mucosa of wakame-fed pigs. Four "wakame assimilative phenotypes" were defined according to the ability of *L. salivarius* FFIG strains to efficiency of assimilating the saccharide in developed wakame broth.

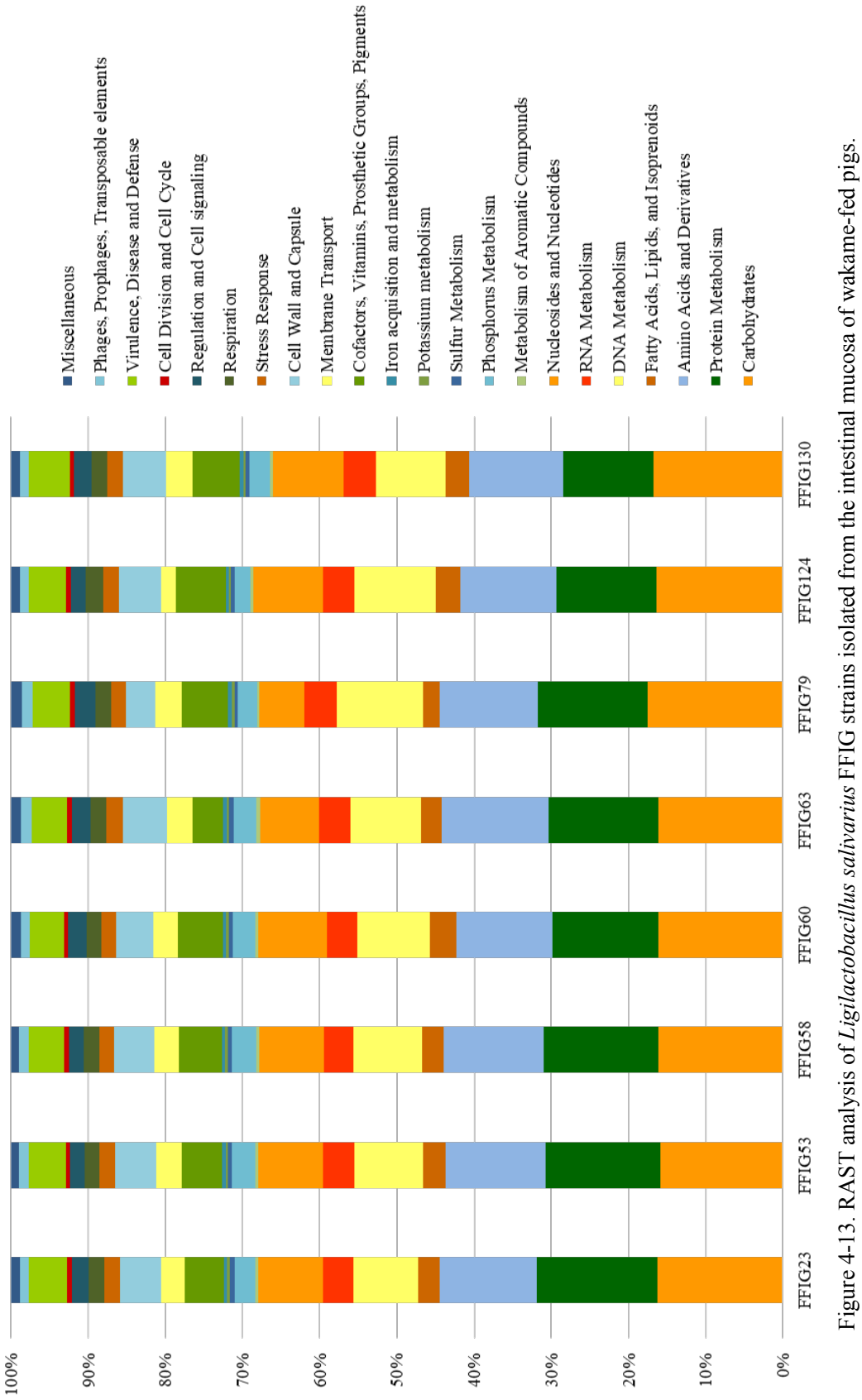


Figure 4-13. RAST analysis of *Ligilactobacillus salivarius* FFIG strains isolated from the intestinal mucosa of wakame-fed pigs.

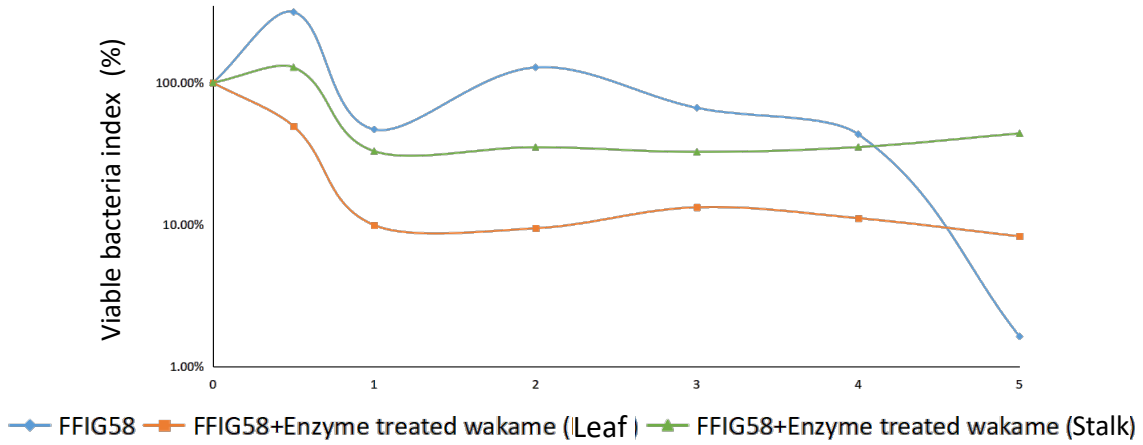


Figure 4-14. Viable bacteria index of *Ligilactobacillus salivarius* FFIG58 when stimulated in simulated gastric juice. Samples were taken at 0, 0.5, 1, 2, 3, 4, 5 h. Viable bacteria index was measured compared to viable bacteria counted at 0 h.

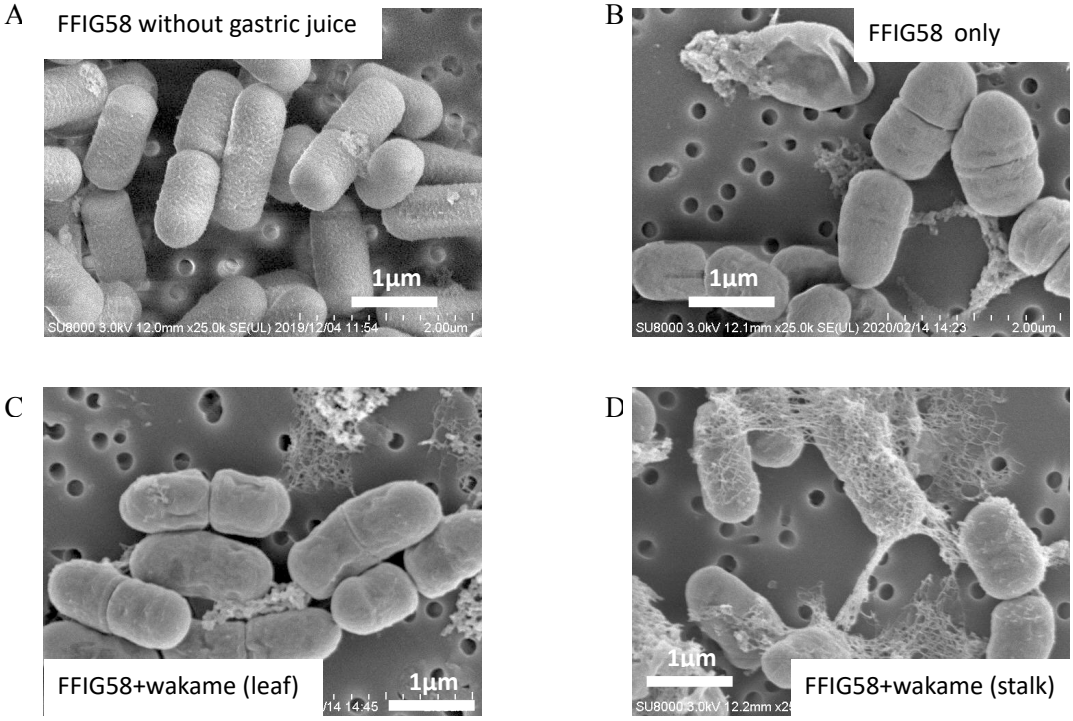


Figure 4-15. Photos by scanning electron microscope (SEM) of *Ligilactobacillus salivarius* FFIG58 fermented in MRS (A), stimulated in gastric juice after 5 h (B), stimulated in gastric juice with leaf wakame after 5 h (C), stimulated in gastric juice with stalk wakame after 5 h (D).

4.4 Discussion

The cell wall polysaccharide of wakame is constituted mainly by hemicellulose and cellulose. In addition, wakame contains several bioactive polysaccharides including alginic acid, fucoidan (sulphated fucose), sargassans, and laminarin (β-1, 3 glucan)[100]. It has been shown that the hydrolysates of some brown algae had a mixture of monosaccharides, including galactose, glucose, mannose, fructose, xylose, fucose, and arabinose[101]. In this work, we treated wakame with two enzymes for the preparation of broths: cellulase and hemicellulase. Thus, it was speculated that the saccharide in enzyme-treated wakame broths could be glucose, xylose, arabinose, mannose, galactose, the sulfated form of those monosaccharides or cellobiose. The result of TLC analysis showed that only one monosaccharide and one disaccharide were present after the treatment of wakame with the mentioned enzymes. The R_f value of monosaccharide was similar to glucose and fructose (Table 4-4), which indicates the possibility that the monosaccharide may be glucose attached with some radical groups, such as sulfate. On the other hand, our results allow us speculating that the disaccharide may be cellobiose due to the characteristics of the enzymes used in this work to treat the leaf and stalk wakame powders.

Porcine intestinal *L. salivarius* strains were able to utilize the saccharides present in both wakame broth treated with enzymes. However, lactobacilli strains were not capable to metabolize and utilize the wakame broths with no enzyme

treatments (data not shown). It was reported that a *L. plantarum* strain isolated from musts and wine had the ability to produce β-D-glucosidases[102], indicating that some strains of this species may have the ability to assimilate cellobiose. Thus, we selected several *L. plantarum* strains from our lactobacilli library in order to evaluate their capacities to grown and assimilate wakame mediums. The results showed that *L. plantarum* strains could utilize saccharides in both leaf and stalk wakame broths in a more efficient way when compared to the *L. salivarius* strains (Fig. 4-9). These results open an interesting line of research for the near future dedicated to perform further studies with *L. plantarum* strains with non-enzyme treated wakame broth. If a *L. plantarum* strain with a high ability to efficiently initiate the degradation of wakame polysaccharides is found, the use of enzymatic treatments to promote the growth of *L. salivarius* strains in wakame based mediums could be avoided. Then, an immunosymbiotic feed based on wakame and two lactobacilli strains (*L. plantarum* and *L. salivarius*) could be a real alternative.

To the best of our knowledge, there are no reports on the ability of *L. salivarius* strains to assimilate wakame, except for the works of our group. However, it should be mentioned that a previous study indicated that *L. salivarius* strains were increased in the intestine of wakame-fed pigs[14]. This study indicated that in the porcine gastrointestinal tract other metabolic pathways are able to release the saccharides that *L. salivarius* strains are able to use. One alternative

could be other bacteria such as *Bacteroides plebeius*, which could degrade wakame[103] into saccharides that can be assimilated by *L. salivarius* strains. The identification of saccharides contained in wakame as well as in wakame-based mediums need to be investigated in more detail. Furthermore, the metabolic pathways used by *L. salivariu*s to grow in wakame-based mediums should be elucidated to generate information that can be useful in the biotechnological application of these strains. Moreover, the ability of different strains to assimilate wakame should be deepened to elucidate if the different metabolic capacities of the strains will impact on the functionality of the immunosynbiotic foods and feeds aimed to be developed. In this regard, the data presented in this chapter indicate a distinct ability of the different strains to assimilate saccharides and grow in wakame-based mediums. Among the evaluated strains, *L. salivarius* FFIG124 and FFIG63 stood out for its ability to grow in stalk wakame-based mediums while FFIG58 and FFIG79 showed a better capacity to grow in leaf wakame-based broths. The comparative genomic analysis highlighted the differences in the set of genes present in each genome and encouraged further studies evaluating the expression of genes involved in saccharides assimilation.

Immunobiotics perform their beneficial effects to host interacting with the cells of the intestine or colon mucosa. For exerting those positive effects, it is important for immunobiotics to survive the severe acidic environment in the stomach. *Lactobacillus* strains are considered to be resistant to acid[104]. However,

it has been shown that the survival of *Lactobacillus* strains in acidic conditions is a species and strain dependent characteristic. In fact, some lactobacilli could be highly sensitive when pH is below 3.0[105] [106]. In the fermentation of lactobacilli where pH raging from 4 to 7, they continued to consume saccharide during the stationary phase and could produce additional ATP for the cell viability[107]. The presence of metabolizable saccharides significantly increased the survival of *Lactobacillus rhamnosus* GG in acidic conditions[108]. On the other hand, glucose extracted from malt, wheat and barley exhibit a protective effect on the viability of *L. plantarum*, *L. acidophilus* and *L. reuteri* in acidic condition[109]. In agreement with those previous studies, the survival of *L. salivarius* FFIG58 was significantly increased with the presence of both leaf and stalk wakame (Fig. 4-11). Moreover, the SEM analysis demonstrated that *L. salivarius* FFIG58 cells were covered with fiber-like components contained in enzyme-treated wakame broth, which could protect the cell from the acidic environment. Then, our results showed that in addition to giving substrates for growing, wakame can offer a protective microenvironment to *L. salivarius* strains improving their survival to acidic conditions.

4.5 Summary

Eight selected *L. salivarius* strains (FFIG23, FFIG53, FFIG58, FFIG60, FFIG63, FFIG79, FFIG124, FFIG130) were allowed to grow in two wakame-based broths containing enzyme-treated wakame leaf or wakame stalk, which were recently developed by our research group. A prolonged fermentation experiment was designed to investigate the consumption of the saccharides in wakame broths. By using TLC analysis, the eight strains were confirmed to have the ability to utilize the saccharides contained in enzyme-treated wakame, although with differences between them. In addition, we demonstrated tha *L. plantarum* strains isolated from pig mucosal tissues also had the ability to assimilate wakame leaf as *L. salivarius.* When grown in enzyme-treated wakame broth, the cell body of *L. plantarum* strains became longer, swelled, and bent; while *L. salivarius* strains had no significant changes on the morphology.

The main monosaccharide present in wakame-based mediums seems to be glucose, which is probably attached to some radical group such as sulfated form. In addition, the disaccharide present in wakame-based mediums may be cellobiose according to the TLC analysis. The identification of saccharides contained in wakame broths need to be further investigated. Moreover, the precise metabolic pathways used by each immunobiotic *L. salivarius* strain for growing in wakame should be clarified in order to efficiently apply them in biotechnological developments.

Wakame was capable of improving the survival of *L. salivarius* FFIG58 in gastric conditions, providing an additional advantage to the provision of substrates for the growth of lactobacilli. These results indicate that wakame is a great candidate as immunoprebiotics for the development of immunosynbiotics.

Chapter 5

Conclusion

In this study, we first evaluated the immunomodulatory ability of *L. salivarius* strains isolated from the intestine of wakame-fed pigs. For this purpose, we used a porcine *in vitro* system originally developed by our research group: the PIE cell line. For the evaluation of lactobacilli´s immunomodulatory capacities, PIE cells were challenged with TLR3 and TLR4 ligands, which are molecular patterns related to viral and bacterial infections, respectively. Based on this screening, eight *L. salivarius* strains were selected for further studies. Comparative genomic studies indicated that cell wall and the surface molecules expressed in the different FFIG strains are involved in their differential immunomodulatory abilities. Among the strain evaluated, *L salivarius* FFIG58 stood out for its remarkable immunomodulatory potential.

In addition, the adhesion to mucins and to intestinal epithelial cells was evaluated for the selected strains. *L. salivarius* FFIG79 had the strongest adhesion ability to soluble porcine mucin while the FFIG58 strain had a remarkable ability to adhere to PIE cells. No differences in bacterial structures were observed when the two strains FFIG58 and FFIG79 were compared by SEM. Furthermore, genomic comparison indicated no evident surface structures that could explain their greater ability to adhere to mucins or PIE cells.

Of note, the comparison of the adhesion factors of the eight strains and their immunomodulatory capabilities allowed us to conclude that there is no

correlation between the immunomodulatory and the adhesion abilities in the *L. salivarius* strains.

The eight *L. salivarius* strains were able to assimilate enzyme-treated wakame, although differences were observed between the strains. Genomic comparison indicated that these differences could be related to set of glycosylhydrolases genes as well as other genes involved in the catabolism of saccharides that are present in each genome. Furthermore, a difference between species could also be observed, since the strains belonging to the genus *L. plantarum* were more efficient to assimilate wakame than *L. salivarius* strains. In addition to providing substrates for growing, wakame exerted a protective effect by increasing the viability of bacteria under stimulated gastric conditions.

The results of this work highlight the promising possibility of the combination of potential probiotic/immunobiotic *L. salivarius* strains and wakame in the development and application of functional feeds or foods as substitutes of antimicrobials against infectious diseases, in both humans and animals. This thesis work represents the first step in the development of immunosymbiotics that help to fight infections.

Future *in vivo* studies including animal models and clinical trials may lay the scientific bases for applying wakame-based immunosymbiotics to prevent gastrointestinal infections. Furthermore, the recent advances in the understanding of the cellular and molecular mechanisms involved in the improvement of anti-

infectious defenses by beneficial immunobiotic microorganisms clearly indicate that they can exert their beneficial effects not only locally in the intestinal mucosa, but in addition they can influence immunity in distal mucosal sites like the respiratory tract[110]. Then, the evaluation of the ability of wakame-based immunosynbiotics to modulate immunity in distal mucosal sites is an interesting topic for future research, since a functional food having this characteristic could help in the combat against respiratory pathogens such as the new SARS-CoV-2 virus[110].

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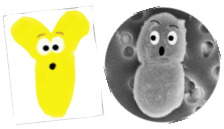
皆さん、ご協力ありがとうございました。



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2021. 2. 4

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